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MSS. for No. 5 should be in the hands of the Editor before August 1st. Contributors are specially requested to arrange references in the manner uniformly adopted in this Journal.

OBITUARY NOTICE

R. A. A. TIGERSTEDT.

(28 Feb. 1853—2 Dec. 1923.)

In the late Prof. R. A. A. Tigerstedt physiology has lost one of its most active and endowed workers, whose influence extended far beyond his native country.

Tigerstedt was born in Helsingfors (Finland). He studied in the University of Helsingfors, where he took his Ph.D. in 1873 and his M.D. in 1881. In the same year he was appointed Privat-dozent (lecturer) in physiology. Soon after this he accepted the post of assistant in Physiology at the Karolin Medical-Surgical Institut in Stockholm under Prof. Ch. Lorén. In 1886 he obtained the professorship in this Institute and in 1900 accepted the Chair of Physiology in the University of Helsingfors. He resigned this post in 1919, his son Carl Tigerstedt taking his place.

The work of Tigerstedt lay along many different lines. His early researches were chiefly concerned with nerve and muscle physiology (*Mitt. von physiol. Labor. des Carol, M.-Ch. Instituts*, herausgegeb. von Ch. Lorén, Stockholm, 1882–86). In Ludwig's laboratory in Leipzig he started working at the physiology of circulation and continued the investigation of this problem nearly until his death (1917: *Die Veränderungen des Blutdruckes nach den Vagusstillstand*). Very important investigations were performed in this field (*e.g.* the work with the "Stromuhr" etc.). Tigerstedt's work on metabolism and nutrition, which he began in 1895, is also well known (*e.g.* Tigerstedt-Zondén's respiration chamber). Some of his investigations concern the sense organs and animal heat.

Tigerstedt's literary work also exerted a considerable influence on physiologists. The famous *Lehrbuch der Physiologie des Kreislaufes*, Leipzig, 1893 (2nd ed. in 4 vols. just published), not only gave a full review of the literature, but was an example of a true scientific criticism. Many articles were written by him on different problems of physiology in the *Ergebnisse der Physiologie*, *Nagel's Handbuch der Physiologie*, *Winterstein's Handbuch der vergleichenden Physiologie*, etc.

The *Lehrbuch der Physiologie des Menschen* (10 editions, from 1897 till 1923) enabled many thousands of German, English and Russian students (the Textbook was translated into English and Russian) to study physiology from a man whose knowledge was gained at first hand: he was an exceptional book lover and every paper he quoted he read in the original.

Everybody who knew Tigerstedt personally appreciated in highest degree his generous and true heart[1].

B. B.

[1] Particulars of the life and work of Tigerstedt are to be found in C. G. Lantesson's article in *Skand. Arch. Physiol.* 1924, **45**, 1–6.

LXV. ON THE CULTURE OF THE MARINE DIATOM *NITZSCHIA CLOSTERIUM* (*F.*) *MINUTISSIMA*, IN ARTIFICIAL SEA-WATER.

By EDITH ANNIE PEACH and JACK CECIL DRUMMOND.

From the Biochemical Department, Institute of Physiology, University College, University of London.

(*Received March 12th, 1924.*)

It was shown by Miquel [1892] that the culture of diatoms, either fresh-water or marine, may be successfully carried out in the laboratory if certain nutrient elements, present in the form of salts, are added to the water, sea-water or distilled. These elements are nitrogen, phosphorus, sulphur, potassium, calcium, magnesium, iron, silicon, sodium, bromine and iodine. No particular stress was laid on the necessity for the last three though they were always added. Miquel, moreover, emphasised the necessity for the addition to the medium of some organic nutrient such as sterilised macerations of bran or straw, though this is not in accordance with the general idea of the metabolism of green plants. Allen [Allen and Nelson, 1910] stated that the omission of the addition of such infusions to natural sea-water did not affect the amount of growth obtained in the case of the plankton diatoms. He showed [1914], however, that good cultures of the marine plankton diatom *Thalassiosira gravida* Cleve, cannot be grown in an artificial sea-water even with the addition of nitrates, phosphates, and iron. The artificial sea-water was made by dissolving in doubly distilled water Kahlbaum's pure chemicals in the proportions in which the salts occur in natural sea-water. The bromine and iodine ions were, however, replaced by an equivalent amount of the chlorine ion. The addition of 1 % natural sea-water, and in some cases less, produced excellent cultures. A number of substances both organic and inorganic, the latter including potassium bromide and iodide, were added to the artificial medium but no substance was found to replace the 1 % natural sea-water. On the evidence that the active principle appeared to be destroyed by heat, Allen suggested that it was probably of an organic nature. Such a factor would appear to be comparable with the "auximones" postulated by Bottomley [1914 and later papers]. It was with the object of identifying and isolating this substance that the work reported in this paper was undertaken.

Experimental.

The procedure adopted in the preparation of the artificial medium, and in the culture of the diatoms, was, with a few modifications, essentially that described by Allen and Nelson [1910]. The water used in the preparation of the artificial sea-water was prepared in the following way. Distilled water from an ordinary copper-still was refluxed with alkaline potassium permanganate for 24 hours, filtered through an ordinary filter-paper to remove any manganese dioxide, and re-distilled in an all-glass distilling apparatus fitted with two Kjeldahl traps to prevent any mechanical carrying over of substances in the form of spray into the distillate. The first 100 cc. of each distillate was always rejected. Very great care was taken to protect this water from contamination by dust particles in the laboratory air. Glass or silica apparatus, freed from possible organic matter by boiling in chromic acid, was used throughout the experiments. All vessels thus cleaned were rinsed thoroughly with ordinary distilled water and finally with the water prepared as above.

The chemicals used in the preparation of the artificial sea-water were either Kahlbaum's chemicals "Pure for Analysis," or "Analytical Reagents" from the British Drug Houses.

The composition of the sea-water used by Allen was based on Dittmar's analyses in the "Challenger Reports" and was as follows:

	Cc. of molecular solution contained in one litre	G. per litre		Cc. of molecular solution contained in one litre	G. per litre
NaCl	480·80	28·13	$MgCl_2$	26·70	2·55
KCl	10·28	0·77	$MgSO_4$	29·06	3·50
$CaCl_2$	10·86	1·20	$NaHCO_3$	1·25	0·11

The artificial sea-water used by us in this work was prepared in these proportions with the exception of the sodium bicarbonate, of which 0·22 g. per litre was used in accordance with Allen's observation that increased alkalinity is favourable to diatom growth. This observation has been confirmed by us. Approximately molecular solutions of calcium and magnesium chlorides were prepared and their exact strength determined volumetrically. These solutions were kept sterile in glass-stoppered flasks. Whenever the flasks had been opened they were always re-sterilised by being heated slowly to 100°, with the stoppers loosely inserted, stoppered, and the stoppers tied down with sterilised parchment. By this means the two stock solutions were kept sterile with very little change in concentration. It had previously been observed that filamentous growths appeared in unsterilised solutions, even of molecular concentration, kept at 1–2° for some weeks. These were either of a siliceous or fungal origin [Moore, 1915]. Hence it was deemed advisable to avoid the use of stock solutions wherever possible. With the exception of the calcium and magnesium chlorides, which could not be weighed out accurately, the dry salts for each litre of artificial sea-water were weighed out as required.

To the artificial sea-water was now added Allen's modification of Miquel's two nutrient solutions.

Solution A.	KNO_3	20·2 g.
	Distilled water to 100 cc.	
Solution B.	$Na_2HPO_4 . 12H_2O$ ...	4 g.
	$CaCl_2 6H_2O$	4 g.
	$Fe_2Cl_6 12H_2O$—melted ...	2 cc.
	HCl (pure, concentrated)	2 cc.
	Distilled water	80 cc.

These solutions also were kept sterilised. To each litre of artificial sea-water 2 cc. of solution *A* and 1 cc. of solution *B* were added, and the whole was then boiled gently for 15 minutes, when the precipitate of ferric, calcium and magnesium phosphates flocculated, and settled to the bottom of the flask. This precipitate was removed by careful decantation; we have confirmed Allen's statement that if this precipitate is removed by filtration through ordinary filter-paper the filtrate will no longer support diatom growth. No information as to the cause of this has been obtained; it is thought to be due to some toxic substance in the filter paper. The precipitate may be successfully removed by filtration through glass wool.

Following Allen's technique, the cultures were made in "carbon dioxide" flasks of 100–150 cc. capacity; the necks of the flasks were kept covered with closely fitting inverted crystallising dishes. Experiment has shown that the chance of contamination from the outside air of cultures kept under these conditions is small. About 50 cc. of liquid was placed into each sterilised flask and cover and the whole re-sterilised at 95° for 15 minutes on three successive days, with little change in concentration. Inoculation was carried out by means of a glass pipette drawn down to a fine capillary and delivering 0·01 cc. Throughout this work the usual precautions were observed to prevent contamination of cultures by bacteria and moulds.

Inoculations of *Nitzschia closterium* (*f.*) *minutissima*, made into artificial sea-water with the addition of the nutrient salts as described above, gave excellent cultures which were as good as, and, in a few cases, better than the control cultures in natural sea-water which had also been treated with Miquel's solutions *A* and *B*. The cultures in artificial sea-water contained 0·02 % of natural sea-water introduced at the inoculation, and therefore growth might have been due to Allen's factor. In order to reduce the concentration of natural sea-water to a negligible degree the following series of experiments have been repeatedly carried out.

A number of flasks containing the artificial medium were prepared and inoculated; immediately afterwards and from the same parent culture an equal number of flasks containing the nutrient medium prepared with natural sea-water were similarly inoculated. The flasks were observed daily. At the end of one week in summer definite growth had appeared in all the flasks, which

were kept on a table in front of a window facing north. Two weeks later there was heavy growth in all the flasks. Each culture was then sub-cultured into a fresh sample of its own medium and the growth again carefully watched. Similar procedure in sub-culturing was carried out a second, third, and fourth time. Although certain divergences from the average amount of growth in flasks of both series were noticed from time to time, the cultures in the artificial medium could never be distinguished from those in the medium which had for its basis natural sea-water. Good growth was always obtained in all the flasks. It is to be pointed out that inoculation with 0·01 cc. of a parent culture does not ensure the transference of a constant number of organisms. This might possibly account for slight differences in the amount of growth obtained.

We have not therefore obtained any indication of the presence in natural sea-water of a specific substance, essential to the vigorous growth of the diatom used. If there be such a compound, other than silica and the usual salts necessary for plant growth, then it must have been derived in our artificial medium either from the salts used in its preparation or from the atmosphere. This does not exclude the possibility of the "allelocatalytic effect" as designated by Brailsford Robertson [1923] being present, although Cutler and Crump [1923] failed to obtain this effect with a culture of *Colpidium colpoda*. It is to be pointed out that extreme precautions were always taken to avoid contamination of the medium by organic matter even from the dust of the air. The salts used were always the purest that could be obtained though they were not further purified by us.

One further experiment was carried out with regard to the possible existence in sea-water of such a substance specific to the growth of *Nitzschia*. As already stated, Allen, who postulates its existence for the growth of *Thalassiosira* suggests that it is destroyed by heat. To test this, 17 g. of powdered sea-salt, made by evaporating natural sea-water, was heated strongly over a blow-pipe flame in a covered silica crucible for 15 minutes. This amount of salt corresponds to half a litre of sea-water. The bulk of the fused salt was removed by means of a platinum spiral; the fused salt was dissolved as far as possible in the specially prepared water; pure concentrated hydrochloric acid was then added at intervals 0·01 cc. at a time until the solution gradually became quite clear. It was then diluted to 250 cc.; half of this volume was then titrated to neutrality with $N/50$ caustic soda made from pure caustic soda and the "organic-free" water. An equal volume of caustic soda, actually 11·0 cc. was added to the remaining 125 cc., the solution diluted to 250 cc., the nutrient solutions added, the whole boiled to flocculate the insoluble phosphates, the p_H adjusted to 8·0, and the medium sterilised. Great care was taken throughout the various operations performed to avoid contamination of the liquid. This medium gave equally good cultures of the diatom *Nitzschia* as the two control media made from natural and artificial sea-water respectively.

The culture used was a pure one in that it contained only one species of

diatom, it contained however certain common bacteria (*Staphylococcus albus*, *Staphylococcus aureus*, and *B. subtilis*) and moulds, though the latter were very rare. It is obvious that work of this nature should be undertaken with a culture containing absolutely no other organism than the one concerned. Miquel claimed to have prepared cultures of diatoms in a state of absolute purity; Allen has shown the difficulty of such a task and did not succeed in obtaining such a culture. So far our attempts have been unsuccessful though we have been able to obtain parent cultures for inoculation where the number of bacteria present per cc. has been extremely small, of the order of 200 per cc. This has been accomplished by washing the cultures with sterile sea-water in a tall narrow sterilised vessel. The diatoms, which are relatively heavy, easily sink to the bottom of the vessel. Bacterial counts performed on healthy cultures have shown us that the bacterial contamination was extremely small. The failure to prepare an absolutely pure culture of the diatom concerned invalidates the conclusion that the diatom used is definitely autotrophic, as Bottomley [1914] states that *azotobacter* has the power of elaborating products which greatly increase the growth of *Lemna* plants, which cannot thrive in a culture containing only mineral salts. It seems improbable, however, that the bacterial contamination in our cultures was ever great enough to act in this way. Further investigation is being carried out with reference to this point.

Summary.

No evidence has been obtained of the existence in natural sea-water of an organic substance specific to the growth of the diatom *Nitzschia closterium* (*f.*) *minutissima*. This organism appears to be able to grow and thrive in an inorganic culture medium.

Our thanks are due to Dr Allen for the culture of the diatom used, and for supplying us with much very useful information.

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LXVI. CHANGES IN THE CHEMICAL COMPOSITION OF THE TISSUES OF THE HERRING IN RELATION TO AGE AND MATURITY.

By JOHN RONALD BRUCE.

From the Marine Biological Station, Port Erin.

(*Received March 17th, 1924.*)

Numerous proximate analyses of the tissues of the herring and other clupeoid fishes have been published from time to time. Milroy [1906, 1907, 1908] and Johnstone [1915, 1918, 1919, 1920] have established the fact that herrings caught at different times and in different localities vary widely in composition, especially in fat-content, and they have correlated these variations with the reproductive phases and periods, and with the bodily measurements of the fish. Up to the present, however, no attempt has been made to ascertain the extent to which age, as distinct from maturity or size, affects the chemical composition of the tissues. This is doubtless due to the fact that no reliable and universally applicable criterion of age has been available. Length, and other standardised measurements, may afford fairly reliable evidence as to the relative ages of the individuals in a single shoal, but the existence, within the species, of separate "races" of fish, of widely differing characters, invalidates any comparison of data obtained from other and distant shoals.

It is now widely believed that the number and distribution of the so-called "winter-rings" upon the scales of the herring are a reliable guide to the age of the fish, and upon this basis the samples used in this investigation have been classified. (Since the methods of scale-reading are probably not widely known outside the circle of fishery research, a note descriptive of the process has been added at the end of the paper. It must be admitted that the validity of the method still lacks final proof; so far, however, the data obtained by tissue-analysis have been corroborative.)

During the fishing-season (June—September) of 1923, analyses were made of a series of herring samples taken from the Peel (Isle of Man) landings, each sample having been selected by Mr W. Birtwistle, of the Oceanography Dept. of the University of Liverpool, so as to be homogeneous as regards age and sexual maturity. This necessarily involved a scale-reading and a determination of the gonad-condition on the Hjort scale, upon each of the 156 fish

analysed. Mr Birtwistle's invaluable co-operation, most gratefully acknowledged, made it possible to examine samples of fish in their third, fourth and fifth years, and ranging through successive stages of gonad-development from maturing virgins to "matties" and "spents."

Briefly, the results of this part of the investigation may be summarised by saying that age, considered as a separate factor, appears to have much the same influence upon the chemical composition of the muscle-substance as sexual maturity, also separately considered. In other words, individuals in the same stage of gonad-development, but differing in age, show a sequence of water/fat ratio, and nitrogen-content, similar to that of individuals in successive stages of gonad-development but presumably of the same age. The older, and therefore larger, herrings, with full roe or milt, have a higher calorific and food-value than younger, even if mature fish.

Included in this paper, also, are the results of some analyses of herrings from the Clyde Area, made in 1922, by Mr John Secker, B.Sc., sometime Biochemical Assistant at the Millport Marine Station. For the use of these data, hitherto unpublished, I am indebted to Mr R. Elmhirst, F.L.S., and the Scottish Marine Biological Association, and personally to Mr Secker, for various explanatory notes. In carrying out these latter analyses, special attention was paid to the possible rôle of phosphorus in the tissue-changes involved in the sexual cycle. The results are of value, in that they establish further general data as to the variations in the proximate food-materials, but it is clear that much further and more detailed work will be necessary before it is possible to advance any general theory as to the part played by phosphorus and the lipins in the history of fat-metabolism and translocation.

Finally, my thanks are due to Prof. Jas. Johnstone, D.Sc., and Mr W. C. Smith, respectively Director and Curator of the Port Erin Station, for helpful advice and assistance.

Analytical Methods.

[*The following descriptions apply only to the Port Erin samples, unless otherwise noted.*]

The number of fish taken for an analysis was generally determined by the supply available—each sample consisting of fish of a single age-group (as determined by scale-rings) and, so far as possible, in the same stage of maturity. This entailed much labour in the selection of the samples from the catch as landed, and occasionally very small numbers only were obtainable. A sample of ten fish was aimed at, but some few samples were as small as four, while one included 15 fish; the mean number per sample was eight. Samples were duplicated, from time to time, throughout the season. The fish were sent to Port Erin by rail from Peel, I.O.M., and in every case the weighed portions for analysis were being dried within twelve hours after the death of the fish.

Preparation of samples for analysis. The fish, forming a homogeneous sample, were wiped free from adherent slime and loose scales, and two parallel cuts were made, about 1 cm. apart, down the full length of the side of the fish, from "shoulder" to tail. The strip of skin, so isolated, was picked up by forceps at the shoulder of the fish, and stripped off, with its attached scales—this operation was perfectly easy, and exposed a long narrow strip of muscular tissue. The point of a dissecting-knife was then inserted between the strip and the backbone, and while the strip was raised by forceps, it was freed from the bone along its length. In this way strips were taken from each fish comprised in the sample—one strip from each if there were eight or more fish, or two strips, one from each side, if the sample was a small one. The strips were laid side by side on a clean plate, and then, by drawing a sharp knife transversely across them all, small blocks of tissue, each about 0·5 cc. in volume, were separated from the shoulder, middle, and tail regions of each strip; these were lifted by forceps, and dropped into a dried and weighed extraction thimble (22 × 80 mm.) supported in a dry tared weighing bottle. It is necessary to sample the tissue at different points along the length of the fish, as there is some evidence that the fat, at any rate, is unequally distributed throughout the muscles [Johnstone, 1918; Milroy, 1908]. In this way, a fair sample of the edible muscular portion of each fish was represented in the material analysed.

In the case of the Scottish samples, the sexes were analysed separately, but when dealing with the Irish Sea fish no distinction of sex was made, since previous work indicates that the difference between the muscle-substance of the sexes is small and inconstant.

The composite sample taken for analysis was, roughly, 10 g. in each case.

During the entire process, there was never any visible loss of oil from the tissue, even in the comparatively fat-rich summer samples. Johnstone [1918] emphasised the care necessary to prevent loss of oil during sampling, which he effected by removing from each fish a series of thin transverse slices. This method was followed in the case of the Scottish samples, but it is not thought that any differences between the analytical results are attributable to this cause. Milroy [1906, 1908] passed the whole of the muscular substance of each fish through a fine mincing machine, afterwards removing portions for analysis from the pulped mass; it seems hardly possible that some loss or, at any rate, separation, of oil should not have occurred during this process.

Estimation of Water. The extraction thimble, containing about 10 g. of wet tissue, prepared as above, and supported in a weighing bottle, was placed in a steam-oven, maintained at 96°–98°. Consecutive weighings were constant after about 18–20 hours at that temperature, though in practice, the samples were dried for at least 24 hours, before the two check-weighings, to the nearest mg., were made. It is not claimed that every trace of water was driven off under these conditions, but the method was convenient, and gave consistent results; in view of the comparatively large errors involved in sampling, it was

not thought justifiable to aim at the highest precision in this, or in the subsequent operations.

The error introduced by oxidation of fatty substances, at the temperature of the drying process, to which attention has been drawn by Atwater and Woods [1906], has been investigated by Johnstone [1918], who has shown that the resulting increase in weight need not be taken into consideration when the values sought are intended only for general or dietetic purposes. It will be seen, however, on reference to the following section, that the error which may be introduced by oxidation of the fats, at another stage of the analysis, is by no means negligible.

Estimation of Fat. The term "fat" is here used as synonymous with "carbon tetrachloride extract," in the comprehensive sense in which it is usually employed in food-analyses, since phosphatides and possibly other substances are extracted with the true fats, and weighed together with them. Milroy [1908] extracted the dried tissue, in Soxhlet's apparatus, first with alcohol, and afterwards with ether, but does not mention the amount, if any, of the alcoholic extract. The extractions of both the Port Erin and Scottish samples, following Johnstone's method [1915, 1918], were made with pure carbon tetrachloride (B.P. 76°·7). The thimble, lightly plugged with glass-wool, was placed in a 100 cc. Soxhlet extractor; 150 cc. of carbon tetrachloride were placed in the receiving flask, a small portion being used to rinse traces of adherent oil from the weighing bottle. Extraction was allowed to proceed in the usual way for about two hours, with the siphon operating every five minutes. The extracting solvent was quite colourless after the third or fourth siphoning, even in those cases where the first extract was dark brown.

After extraction, the bulk of the solvent was recovered by distillation, and the flask containing the dark oily residue was placed in the steam-oven and dried to constant weight, this requiring as a rule about 6–8 hours. As a check, the thimble, with its fat-free contents, was also dried to constant weight, and the loss of fat ascertained by difference. As a result, it was found that the weight of extracted fat was invariably greater than the loss sustained by the dried tissue, the mean difference, calculated on 18 samples of the Port Erin series, amounting to 1·96 % of the absolute weight of fat present. It should be noted, however, that the fat-content of the original tissue, calculated on the basis of the carbon tetrachloride extract, exceeds only by 0·3 % that calculated upon the mean loss in weight of the extracted tissue. The latter value is believed to be the more reliable, and is that reported in the analytical tables.

Since the drying was most carefully conducted, and final constancy of weight established, there can hardly have been any appreciable retention of carbon tetrachloride by the oily residue, and the difference must be largely, if not wholly, accounted for by oxidation of the free fat. The stage at which such oxidation occurs, however, is not clear, for when control experiments were made, drying the extracted oil in a slow stream of CO_2 led in through

the tubulure of the steam-oven, no improvement was noted beyond a slight speeding-up of the drying process. The precaution was always taken, two or three times during the period of drying, and before weighing, to blow the heavy carbon tetrachloride vapour out of the flask by a jet of air from a foot-bellows. A quantity of the carbon tetrachloride, as used for the extractions, was boiled for some hours under a reflux condenser, then distilled off, and the flask dried in the oven and weighed, exactly as in the estimations—there was no ponderable residue.

Estimation of Protein. The usual Kjeldahl method was followed, with a few slight modifications dictated by convenience. In a 250 cc. Kjeldahl flask were placed 0·5 g. of the powdered, dry, fat-free material; 10 g. of potassium sulphate, and 20 cc. of pure conc. sulphuric acid; to accelerate oxidation, 0·3 g. of crystallised copper sulphate was added. After boiling gently for 2–2½ hours, the then clear solution was cooled, and diluted with 50 cc. of distilled water. The flask was provided with a rubber stopper, tap-funnel and splash-head, the latter connecting up with a vertical condenser, of which the lower end dipped below the surface of 75 cc. of $N/10$ sulphuric acid, containing a few drops of methyl red. A fragment of zinc was dropped into the dilute acid mixture in the flask, then, immediately connecting up, an ample excess (100 cc.) of 40 % caustic soda solution was rapidly run in through the tap-funnel. The alkaline mixture was boiled for at least 30 minutes, no trouble from "bumping" being experienced. The back-titration was made with $N/10$ NaOH, the end-point being quite sharp. As a rule, about 50 cc. of the acid were neutralised by the evolved ammonia, each cc. corresponding to 0·0014 g. of "total nitrogen."

There is some uncertainty as to the applicability of the usual factor ($\times$ 6·25) when converting total nitrogen into *fish* protein. This is discussed by Atwater [1892] and Johnstone [1918], the latter inclining to the view that the estimation of protein by difference—*i.e.* assuming that whatever is not water, nor carbon tetrachloride extract, nor non-volatile mineral matter ("ash"), is protein—is good enough for general and dietetic purposes. It is not, however, accurate enough for metabolic studies, in which the variations in the amount of nitrogenous but non-protein substances, throughout the seasonal and sexual cycle, require to be established. It is quite certain, moreover, that a small though variable proportion of the nitrogenous substances present in the fresh tissue is carried through with the fats during the carbon tetrachloride extraction, in the form of phosphatides (lecithin, etc.). To ascertain the possible magnitude of this source of error, Kjeldahl determinations were made upon four samples of fish from the Clyde area, using, in each case, the fresh moist material and the dry, extracted residue. Expressed in both cases as a percentage of the original wet weight, the protein ($N \times 6{\cdot}25$) in the former is 1·5 % greater than that in the latter. It may be noted, in this connection, that Johnstone [1919], while examining canned herrings, found that the carbon tetrachloride extracts contained from 1·0–1·62 % of "proteid."

It would seem, therefore, very desirable that the Kjeldahl estimations should always be made upon the fresh tissue; owing, however, to the necessity of dealing rapidly with the samples as obtained, it was usually found impossible to do this, and the dried, fat-free material was set aside for nitrogen-determination at a later date. Until such time, therefore, as it is possible to determine the nitrogen-content of every carbon tetrachloride extract, and to make the necessary correction, the protein-figure must be accepted with the reservation that it is somewhat too low, from this cause, and that in any case, the 6·25 factor may not be strictly correct. That both causes operate in the same direction may be seen from the variations in the percentage of nitrogen calculated upon the dry, fat-free, ash-free tissue-residue; these values are appended to the table of analyses (Table II), in the column "N." They are invariably less than 16, which is the mean percentage assumed when the factor $\times$ 6·25 is used. The mean value of "N," for 17 samples, is 14·5, whence the appropriate Kjeldahl factor is $100/14{\cdot}5 = 6{\cdot}9$. The extent to which the values of "N" diverge from the standard figure is an indication both of the varying nature of the tissue-proteins and of the amount of nitrogenous but non-protein matter which goes to make up the total of the dry, extracted, and ash-free muscle substance.

Estimation of "Coagulable Protein." Estimations of the coagulable protein were made only upon the muscular tissue of the Scottish samples. The method followed was that of Milroy [1907]. 5 g. of the composite sample of fresh muscle were ground in a mortar with 5 g. of anhydrous sodium sulphate; 100 cc. of methylated spirit were added, and the mixture boiled under a reflux condenser for three-quarters of an hour. The spirit was then decanted off, and the coagulated residue washed with successive quantities of boiling water, until the washings were free from sulphate. It was then submitted to the usual Kjeldahl estimation.

Estimation of non-volatile Mineral Matter. This was determined by igniting about 0·5 g. of the dry, fat-free residue in a small porcelain dish. After heating to dull redness for some time, a few drops of conc. nitric acid were added to the dark ash, which became white on re-heating. It was impossible, of course, to avoid including a few small bones, when sampling the herring flesh, and these tend to raise the ash-content; again, a certain, if small, proportion of the mineral matter consists of chlorides and other volatile salts—these are lost to the analysis; finally, a trace of phosphorus in organic combination may be carried away during the estimation of the fats. Consequently, the values in the ash-columns represent the amount of non-volatile inorganic matter in the fat-free substance; it is probable, however, that they approximate fairly closely to the true "ash," or mineral-content of the living tissue.

Ash determinations were made only upon the Port Erin samples.

Estimation of Phosphorus. The phosphorus-content was determined, in the case of the Scottish samples only, upon the muscle, gonad and liver, of each of the sexes. Neumann's method, slightly modified, was employed. After

oxidation with conc. sulphuric acid, as in the first stage of the Kjeldahl nitrogen estimation, the mixture was cooled and diluted, nitric acid was added, and 20 cc. of 80 % ammonium nitrate, after which it was heated to about 85°, when excess (about 20 cc.) of 10 % ammonium molybdate was added. After standing for 15 minutes, the ammonium phosphomolybdate precipitate was filtered off through a wad of filter-pulp, using suction; both flask and precipitate were then washed free from acid. The precipitate was returned to the flask, and dissolved in a known excess of 0·5 *N* NaOH. The solution was boiled to remove ammonia, and the excess of alkali titrated with 0·5 *N* H_2SO_4, using phenolphthalein as indicator. Each cc. of 0·5 *N* alkali used to dissolve the precipitate corresponds to 1·268 mg. P_2O_5.

As in the case of the other estimations, the validity of the figure obtained is subject to certain reservations. The phosphatides extracted with the fats, containing as they do about 4 % of their weight of phosphorus, account for a certain, if small, loss of this element, while inorganic phosphates are known to be somewhat soluble in the usual fat-solvents when these are not perfectly anhydrous. Even if it were possible to use and maintain the solvent in a perfectly anhydrous condition, it is very difficult to ensure that all traces of water have been removed from the tissue. The total P_2O_5-content is small, amounting, on the average, to 0·56 % of the wet weight of the muscular tissue. The extent to which the organically combined phosphorus is removable by carbon tetrachloride or other fat-solvent may be judged from a series of seven determinations, which show that, on the average, the P_2O_5-content of the extracted tissue is 26 % less than that of the fresh material, both values being referred to the original wet weight.

Note on Carbohydrate. No estimations of carbohydrate have been made, either on the Port Erin or on the Scottish samples, since the amount present in the tissues of the herring (and in fact of most sea-fishes), whether as glycogen or in some other form, appears to be negligibly small at all seasons.

Stirling [1884] was able to demonstrate but the merest trace of reducing sugars in the livers of "mattie" or filling herrings, and showed that this condition was independent of food-intake. Milroy [1908], while believing that glycogen might vary in amount, along with other substances, during the various stages of reproductive activity, found that the amount present in spent fish was "extremely small." Finally, during the present investigation, tests were made upon the liver of the Scottish herring, in Hjort-stage III, which failed to reveal any trace of glycogen or glucose.

Analytical Results.

I. *Manx Herring.*

Samples and Classification. Particulars of the samples taken for analysis are given in Table I, the reference numbers in the first column being those used in the subsequent analytical tables. Samples were obtained from the

last week in June until the beginning of September, when the local boats engaged in the Manx herring fishery ceased operations. It will be noted that the fish sampled were not all caught on the same ground, those of June, July and August being from the inshore or "low" grounds, while the September catches were made in deeper, offshore, waters. Complete dependence upon available commercial supplies, however, put quite out of the question any selection of a fixed fishing ground. There is some evidence, also [Birtwistle and Lewis, 1922], that the Manx herring shoals of June and July comprise a mixture of sub-types different from that occurring later in the season, but it is not thought that either of these factors is likely to have had any serious influence upon the purely chemical data.

The mean length of the fish, in each sample, is given, as the data may be of use in comparing the present analyses with those of other workers—they are not otherwise made use of.

The age, in years, is simply the number of completed winter-rings on the scales, plus one (*vide* Note, p. 484).

The state of the gonads is given on Hjort's notation:

I. Virgin—fish which have never spawned.
II. Maturing virgins or recovering spents, first stage.
III. Gonads occupying about one-half of the body-cavity.
IV. Gonads occupying about two-thirds of the body-cavity.
V. Full—gonads filling the body-cavity.
VI. Spawning—roe or milt running.
VII. Spent—gonads collapsed.

For the actual purpose of the analyses, however, a broader classification was found to be desirable, especially since many of the samples contained individuals in two successive Hjort stages—four "gonad-groups" have therefore been recognised:

Gonad-group	Description	Corresponding Hjort stages
A	Virgin	I
B	Filling	II, III
C	Full	IV, V
D	Spawning and recently spent	VI, VII

(No samples were obtained representative of Group A.)

Analytical Tables. The analyses of the samples, as carried out in order of date, are set out in Table II. For convenience of reference, the results have been calculated as percentages of both the original weight of fresh muscle-substance, and of the dried material. The only points that call for notice at this stage are, first, the remarkable constancy, under all conditions of age and development, of the sum of fat and water, which lies very close to 80 % of the total fresh weight, and, second, the values in the protein columns, which were obtained by multiplying the total nitrogen, found by Kjeldahl's method, by the usual factor, × 6·25. Probably almost wholly as a result of this procedure, the totals in every case fall short of 100 %, the mean defect being

1·75 %. If the factor × 6·9 be used, as indicated by the mean of the values in the column "N" (*vide* p. 474), the totals will be found to lie very close to the theoretical, in every case. Until the precise amount and nature of the non-protein substances present, however, be ascertained, it will be safer to adhere to the accepted factor. The results, of course, retain their relative significance, whatever factor be used, and none of the other components is determined by difference.

Table I. *Manx herrings—Particulars of samples taken for analysis.*

Ref. No.	Date caught (1923)	Where caught	No. of fish in sample	Mean length (cm.)	Age (years)	State of gonads	Gonad-group
1	27 June	"Inshore"	4	24·0	4	II	B
2	,,	,,	4	22·6	3	II	B
3	29 June	,,	6	23·5	4	II	B
4	10 July	,,	4	25·1	4	IV, V	C
5	,,	,,	4	25·6	5	IV, V	C
6	13 July	,, (Port Erin Bay)	5	—	4	IV	C
7	,,	"Inshore"	6	24·8	4	IV, V	C
8	19 July	,,	10	22·6	3	II	B
9	,,	,,	10	24·1	4	IV	C
10	,,	,,	10	25·6	5	IV, V	C
11	24 July	,,	15	23·3	3	I, II	B
12	,,	,,	11	23·9	4	III, IV	C
13	,,	,,	3	25·1	5	II	B
14	14 Aug.	,,	11	22·6	3	II, III	B
15	,,	,,	11	23·9	4	IV, V	C
16	28 Aug.	,,	10	22·9	3	III, IV	C
17	,,	,,	11	24·1	4	V	C
18	6 Sept.	12 m. W. by S. of "Chickens"	10	23·7	3	V	C
19	,,	,,	11	24·9	4	VII	D

(NOTE. Inshore or "low" herrings were taken within six miles of land, between Peel and the Calf of Man.)

Table II. *Manx herrings—Analyses of fresh and dry muscle-substance.*

Ref. No.	Percentage composition of fresh muscle-substance						Percentage composition of dry muscle-substance			"N" (*vide* p. 474)
(Table I)	Water	Fat	Water + fat	Protein (N × 6·25)	Ash	Total	Fat	Protein (N × 6·25)	Ash	
1	63·5	16·1	79·6	—	1·49	—	44·1	—	4·08	—
2	69·2	7·5	76·7	19·7	1·60	98·0	24·4	64·0	5·20	14·5
3	65·5	12·4	77·9	—	1·74	—	35·9	—	5·04	—
4	65·0	15·6	80·6	16·6	1·19	98·4	44·5	47·4	3·40	14·6
5	62·8	18·1	80·9	16·5	1·06	98·5	48·6	44·4	2·85	14·6
6	65·7	13·8	79·5	17·6	1·20	98·3	40·2	51·3	3·50	14·6
7	67·0	11·9	78·9	18·3	1·08	98·3	36·0	55·5	3·28	14·6
8	67·0	12·4	79·4	17·8	1·26	98·5	37·6	54·0	3·82	14·7
9	64·0	16·3	80·3	16·7	1·31	98·3	45·3	46·4	3·64	14·6
10	61·8	19·4	81·2	15·4	1·15	97·8	50·8	40·3	3·01	14·0
11	65·2	14·8	80·0	16·9	1·37	98·3	42·5	48·5	3·94	14·5
12	65·1	15·1	80·2	16·7	1·24	98·1	43·3	47·8	3·55	14·5
13	65·3	14·4	80·7	16·0	1·18	97·9	41·5	46·1	3·40	14·2
14	67·7	10·6	78·3	18·5	1·51	98·3	32·8	57·2	4·67	14·6
15	65·7	13·6	79·3	17·6	1·20	98·1	39·6	51·3	3·50	14·4
16	65·2	13·6	78·8	18·4	1·43	98·6	39·1	52·8	4·11	14·9
17	63·8	16·2	80·0	16·8	1·11	97·9	44·7	46·4	3·07	14·2
18	65·5	14·7	80·2	17·3	1·35	98·9	42·6	50·1	3·92	15·0
19	63·5	17·5	81·0	15·6	1·44	98·0	48·0	42·7	3·94	14·2

Grouping of Analyses. On proceeding to group the analytical results according to the age and condition of the samples analysed, certain relations at once become evident—for example, that obtaining, in a general way, between age and fat-content. If the entire series be divided into three groups, containing respectively the 3-year, 4-year, and 5-year old fish, regardless of date, sex, and genital maturity, it will be seen that the older fish have the higher fat-content in the muscle-substance.

Age of fish (years)	Fat in fresh muscle %	Number of fish upon which estimate is based
3	12·3	60
4	14·9	79
5	17·3	17

A similar but inverse relation can be shown to exist when the water-content is considered. These results are borne out, and rendered even more striking when the data in Table II are fully analysed, so as to show the tissue-composition of the various age-groups, at corresponding levels of sexual maturity. This is done in Table III, where the mean percentages of water, fat and protein at each stage are shown.

Table III. *Manx herrings—Mean percentage composition of fresh muscle, in successive age- and gonad-groups.*

Age of fish (years)	Gonad-group (*vide* p. 476)	Ref. Nos. (Tables I and II)	Mean % composition			
			Water	Fat	Protein	Water/fat
3	B	2, 8, 11, 14	67·3	11·3	18·2	5·96
	C	16, 18	65·3	14·2	17·9	4·60
4	B	1, 3	64·5	14·3	—	4·51
	C	4, 6, 7, 9, 12, 15, 17	65·2	14·6	17·2	4·46
	D	19	63·5	17·5	15·6	3·63
5	B	13	65·3	14·4	16·0	4·54
	C	5, 10	62·3	18·8	15·9	3·32

Since the proportions of water and fat vary in a complementary manner, the ratio of these values affords the best datum of comparison between the various stages. The mean ratio—water/fat—therefore, is given in the last column of Table III.

These ratios may thus be summarised:

Age of fish (years)	Ratio: $\frac{\%\ \text{water}}{\%\ \text{fat}}$, in successive gonad-groups			
	A	B	C	D
3	—	5·96	4·60	—
4	—	4·51	4·46	3·63
5	—	4·54*	3·32	—

* This slightly aberrant value is based upon a very small sample only, Ref. No. 13.

From this it is clear that, at any given gonad-stage, the relative amounts of water and fat present in the muscular tissue vary with the actual age of the fish, and further, that the older fish, at any rate within the age- and gonad-range covered by the samples examined, are richer in fat than the younger.

This is a fact of considerable importance, not only economically—as bearing upon the dietetic value of the herring, and its suitability for salting, canning, or other conservation-process, at the various stages in its life-history—but also from a bionomic standpoint. The older fish, with their greater fat-reserve, are able more successfully to withstand a temporary period of adverse conditions, to disperse more widely in search of suitable feeding and spawning grounds, and finally, when other conditions permit, to mobilise more readily their tissue-reserves, whether localised in muscle or liver, for the building-up of the genital products, with consequent earlier maturation. It is believed that these suggestions are in consonance with the observations of biologists on the movements and habits of the herring.

It is also evident from Table III that, in any given age-group, the more sexually mature individuals have the higher fat percentage. This relation, of course, has already been shown to exist, in the case of Manx, Scottish, and Welsh herrings, by Milroy and by Johnstone. The results now obtained, however, appear to indicate, in the case of the 4-year old fish, that the water/fat ratio continues to fall, even after spawning, whereas the above-named workers agree in the view that the maximum fat-content occurs some little time before the actual spawning period. As a matter of fact, the present results are not subversive of the accepted view, for had samples of herring been available in the actual process of spawning, or in the stage immediately preceding it, it is certain that a maximum fat-content, and a minimum water/fat ratio, would have been found to lie between the values in question, in columns C and D. As it is, the value 3·63 is dependent upon one sample only (Ref. No. 19), consisting of eleven fish in a spent condition. As Milroy [1907] has suggested, it is possible, though not likely, that these fish had commenced to feed and fatten, after being so largely depleted during the final period of maturation, when the discharge of the ova or sperms, together with enhanced muscular activity, and complete abstention from food, make an unprecedented demand upon the tissue-reserves.

It is important, at this juncture, to emphasise the fact that the variations undergone by the tissue-fat depend upon the seasonal as well as upon the sexual cycle [Johnstone, 1915]. Thus, the great rise in fat content marking the early stages of maturation in the autumn-spawning Manx herring is a reflection of the increased metabolism consequent upon the rising sea-temperature. The winter-spawning herrings of the Clyde area, on the other hand, mature in cooling water, and their maximum fat-content is considerably lower than that of Irish Sea fish. The generally low fat-values recorded in this paper for Manx herring of 1923, however, are largely due to the deficiency of sea-temperature and sunshine during the summer months of that year, as well as to the large proportion of younger "inshore" fish which have made up the bulk of the season's catch.

Leaving now the consideration of the water/fat ratio, a further interesting relation becomes evident when the variations in the protein-content are

examined. The data in Table III may conveniently be summarised as before:

Age of fish (years)	Percentage of protein (N × 6·25) in successive gonad-groups			
	A	B	C	D
3	—	18·2	17·9	—
4	—	—	17·2	15·6
5	—	16·0	15·9	—

From this it is clear that the percentage of protein in the muscle substance falls off with advancing age, and with progressive maturation of the gonads, though the range of variation is much less than that encountered in the case of fat and water. It is not easy to account for these variations. They may, in part, be due to the presence of some substance, varying in nature or amount from time to time, in which the nitrogen-content differs progressively from the 16 % assumed to be present in the "standard" protein; though the fairly constant values of "N" (Table II) calculated for the various stages do not lend support to this view. They may, on the other hand, in so far as they represent actual fact, indicate a conversion from protein to fat, as one stage in the tissue-changes accompanying the development and maturation of the genital products.

The diminution of protein-content in the successive age-groups, concurrently with rising fat-content, may be an expression of the same dynamic equilibrium between these substances.

Finally, the proportion of non-volatile inorganic matter in the various samples has been studied, but no definite relation appears to exist between this value and the age and gonad factors so far considered.

II. *Scottish (Clyde Area) Herring.*

As in the case of the Manx herring, a list (Table IV) is subjoined, giving the date, station and condition of each sample examined.

Table IV. *Clyde herrings—Particulars of samples taken for analysis.*

Ref. No.	Date caught (1922)	Where caught	No. in sample	Mean length cm.	Mean girth cm.	Mean weight g.	Sex	Hjort stage
1 *a*	5 Sept.	Skipness	9	25·6	12·8	140·9	F.	II
1 *b*	,,	,,	4	25·9	13·0	139·8	M.	II
2 *a*	21 Sept.	—	9	25·5	12·8	138·5	F.	III
2 *b*	,,	—	6	25·9	12·6	139·0	M.	III
3 *a*	4 Oct.	Imachar Pt.	12	27·0	13·9	162·0	F.	III
3 *b*	,,	,,	11	28·0	14·3	184·0	M.	IV
4 *a*	18 Oct.	—	10	27·4	13·7	172·6	F.	III
4 *b*	,,	—	15	27·2	13·6	175·0	M.	IV

Although the ages of the fish in the actual samples were not determined, it seems quite clear, from collateral scale-readings made upon fish from the same shoals, that 3-, 4-, and 5-year old individuals were included in the samples. The analyses, as carried out by Mr J. Secker, in order of date, are set out in Table V, from which it will be noted that the sexes have been

separately examined, and that in each case three different tissues—muscle, liver, and gonad—have been selected for analysis.

It will be convenient, at the outset, to consider only the water-, fat-, and protein-content of these tissues, deferring until a later stage the phosphorus-values and their significance.

Table V. *Clyde herrings—Analyses of fresh tissues.*

				Protein (N × 6·25)			Phosphorus (as P_2O_5)		
Ref. No.	Tissue	Water* %	Fat* %	Using* dry fat-free residue %	Using fresh tissue %	Coag. protein %	Using* dry fat-free residue %	Using fresh tissue %	Total* %
1 *a*	Muscle	64·5	16·9	17·5	—	14·1	0·65	—	99·6
	Gonad	70·5	3·6	25·1	—	—	0·50	0·81	99·7
	Liver	63·4	15·9	—	—	—	—	0·73	—
1 *b*	Muscle	63·5	18·7	17·2	—	13·0	0·45	0·59	99·9
	Gonad	76·8	3·7	18·6	—	—	—	0·82	(99·1)
	Liver	63·2	23·1	—	—	—	—	0·58	—
2 *a*	Muscle	63·4	18·6	17·0	—	13·5	0·42	0·56	99·4
	Gonad	67·0	4·0	25·1	—	—	0·71	0·86	96·8
	Liver	66·8	13·4	—	—	—	0·54	0·76	—
2 *b*	Muscle	62·0	20·5	16·6	—	—	0·46	0·57	99·6
	Gonad	76·6	3·8	20·1	—	—	1·12	1·30	101·6
	Liver	61·2	21·2	—	—	—	0·43	0·66	—
3 *a*	Muscle	63·2	18·7	17·3	19·6	14·1	0·47	0·57	99·7
	Gonad	66·8	2·5	25·8	27·4	—	0·65	0·85	95·8
	Liver	70·5	9·5	—	—	—	0·61	0·85	—
3 *b*	Muscle	62·7	19·4	17·3	17·4	13·5	0·38	0·57	99·8
	Gonad	75·3	3·0	22·4	23·2	—	1·02	1·17	101·7
	Liver	67·2	15·3	—	—	—	0·40	0·59	—
4 *a*	Muscle	63·0	18·7	(15·3)	18·6	15·3	0·35	0·56	(97·4)
	Gonad	66·6	2·2	26·3	27·6	—	0·56	0·86	95·7
	Liver	71·4	8·9	—	—	—	0·51	0·76	—
4 *b*	Muscle	60·3	22·4	16·8	17·5	13·3	0·39	0·55	99·9
	Gonad	75·3	3·4	23·1	24·4	—	—	1·17	(101·8)
	Liver	68·9	—	—	—	—	—	0·59	—

* Totals are those of values in columns marked with an asterisk.
Values enclosed in brackets are omitted from calculation of averages.

Table VI. *Clyde herrings—Mean percentage composition of fresh muscle.*

	Hjort stage (p. 476)	Ref. Nos. (Table V)	Mean percentage composition: Water	Fat	Water + fat	Protein* (N × 6·25)	Water/fat
Females:							
	II	1 *a*	64·5	16·9	81·4	17·5	3·82
	III	2 *a*, 3 *a*, 4 *a*	63·2	18·7	81·9	17·2	3·39
Males:							
	II	1 *b*	63·5	18·7	82·2	17·2	3·40
	III	2 *b*	62·0	20·5	82·5	16·6	3·02
	IV	3 *b*, 4 *b*	61·5	20·9	82·4	17·1	2·97

* The protein values are those determined upon the dry, fat-free residue.

Composition of Muscular Tissue. The range of gonad-development covered by the samples is too small to admit of their being arranged in "gonad-groups," as in Table III, but if the analyses of the muscular tissue be placed in order

of the successive Hjort stages, as shown in Table VI, the same general relation between the water/fat ratio and sexual maturity becomes evident, as has already been shown to exist in the case of Irish Sea herring.

It will be noted, moreover, that the relation is independent of sex, the slight disparities between the analyses of male and female tissue lying within the bounds of error inherent in sampling and manipulation. The fact that the water/fat ratios are considerably lower than those observed in the Manx samples (Table III) is doubtless to be associated with differences of race and environment (*vide* p. 479); to this cause may be traced the fact that the sum of the water and fat percentages averages 82·1, in contrast with the 80·0 % "constant," noted in the case of the Manx samples. The variations in the protein values, also, show the same downward trend with advancing development of the gonads.

Composition of Gonads. In Table VII the composition of the ovaries and testes is given, as determined over the same range of development.

Table VII. *Clyde herrings—Mean percentage composition of fresh gonads.*

	Hjort stage (p. 476)	Ref. Nos. (Table V)	Mean percentage composition		
			Water	Fat	Protein* (N × 6·25)
Females:	II	1 *a*	70·5	3·6	25·1
	III	2 *a*, 3 *a*, 4 *a*	66·8	2·9	25·7
Males.	II	1 *b*	76·8	3·7	18·6
	III	2 *b*	76·6	3·8	20·1
	IV	3 *b*, 4 *b*	75·3	3·2	22·8

* The protein values are those determined upon the dry, fat-free residues.

The results are too scanty to enable any definite conclusions to be drawn, but so far as they go, they indicate that the male organs are richer than the female in both water and fat, and correspondingly poorer in protein; it is also evident that a diminution of both water and fat content, and a rise in protein content, is associated with the early phases of maturation in both sexes. No constancy is to be observed in the combined percentages of water and fat.

A comparison of the analyses of muscle-substance (Table VI) with those of the gonads (Table VII) affords no significant evidence which would justify the view that transference of fat or protein takes place from the one tissue to the other. Apart, however, from fatty reserves present in the muscle-substance, a considerable amount of solid or semi-solid fat may be observed, in maturing herrings, investing the intestine and other organs. This—the so-called "mesenterial fat"—may be of great value at a time when the available or ingested food-supply fails to meet the energy- and tissue-requirements of the rapidly maturing gonads. The fat, however, is not in a form capable of immediate translocation, and when rendered available by enzyme-activity, it should be possible to detect its even transitory presence in muscle or liver.

Composition of Liver. From Table VIII it will be seen that the liver is rich in fat, more so in the male than in the female, and that there is a marked

falling-off in fat-content concurrently with the maturation of the genital products. It is not improbable, therefore, that the metabolic demands of the growing gonads are met, in the first instance, by the fatty reserves of the liver, while at a later stage, the stores of mesenterial fat may be called upon. There is no evidence that the fat of the muscular tissue undergoes depletion, until shortly before the time of spawning.

Protein-values are not available, since no nitrogen determinations were made upon the liver.

Table VIII. *Clyde herrings—Mean percentage composition of fresh liver.*

	Hjort stage (p. 476)	Ref. Nos. (Table V)	Mean percentage composition: Water	Fat
Females:	II	1 *a*	63·4	15·9
	III	2 *a*, 3 *a*, 4 *a*	69·6	10·6
Males:	II	1 *b*	63·2	23·1
	III	2 *b*	61·2	21·2
	IV	3 *b*, 4 *b*	68·1	15·3

The Rôle of Phosphorus. The determinations of phosphorus in muscle, gonad, and liver, are few and erratic. Further experimental work, improved technique, and a much wider range of samples are needed before any clear idea can be obtained of the rôle of this element in the herring's metabolism. The results in Table IX, so far as they go, appear to indicate that the developing gonads make a heavy demand for phosphorus, the testes having relatively the higher requirement. This is in agreement with the findings of Milroy [1908].

Table IX. *Distribution of phosphorus in the tissues of the herring.*

		Muscle		Gonad			Liver		
Hjort stage	Ref. Nos.	% P_2O_5 using fresh tissue	% P_2O_5 using dry fat-free residue	% P_2O_5 using fresh tissue	% P_2O_5 using dry fat-free residue	Ratio*: Gonad P_2O_5 / Muscle P_2O_5	% P_2O_5 using fresh tissue	% P_2O_5 using dry fat-free residue	Ratio*: Gonad P_2O_5 / Liver P_2O_5
Females:									
II	1 *a*	—	0·65	0·81	0·50	0·92	0·73	—	—
III	2 *a*, 3 *a*, 4 *a*	0·56	0·41	0·86	0·64	1·53	0·79	0·56	1·09
Males:									
II	1 *b*	0·59	0·45	—	—	—	0·58	—	—
III	2 *b*	0·57	0·46	1·30	1·12	2·28	0·66	0·43	1·97
IV	3 *b*, 4 *b*	0·56	0·39	1·17	—	2·09	0·59	—	1·98

N.B. The percentages are in every case calculated on the fresh (wet) weight of the tissues.

* Based upon the values obtained, using fresh tissue.

A wide disparity is to be observed between the P_2O_5 determinations made respectively upon the fresh and extracted tissues. If the extent of the disparity is regarded as in any way indicative of the amount of phosphorus present as phosphatide, or other fat-soluble compound (p. 475), the suggestion may be hazarded that these lecithin-like bodies play an important part in the phosphorus-metabolism of the herring, and in the up-building of the nucleoproteins of the mature genital products. It is not without significance that the phosphatide-content of the liver, as judged on the above basis, appears to be higher than that of the other tissues.

Note.

Estimation of the Age of Herrings. Scales are taken from each fish, cleaned, and mounted dry on a glass slide. The number of opaque or semi-opaque rings is counted, care being taken to distinguish between the fine concentric markings due to the lamellar structure of the scale, and the real "winter-rings" denoting the years of growth.

No difficulty is usually experienced when the number of rings is less than four or five, but when more numerous, the rings are crowded near the edge of the scale, and considerable experience is needed before accurate counts can be made. When a doubtful scale occurs, it is stained in silver nitrate and examined in polarised light—this materially assists in the elimination of false rings.

It should be remembered that autumn and winter spawned herrings exist for about the first six months of their life without scales, so that the first area on a scale is laid down during the period May to September, which is considered to be the scale-growing period. The naked period is not represented on the scale, and should not be overlooked when estimating the actual age of the fish. A winter-ring only becomes definitely a ring when it is bounded by a summer growth of an appreciable extent, so that by the time this ring (in a one-ring fish) is surrounded by a summer growth the fish is well on the way towards completing two years of life; similarly with older scales the actual age in years is taken as being one more than the number of winter rings on the scale [Birtwistle, 1921; Birtwistle and Lewis, 1923].

Summary.

Analyses have been made upon a series of herring samples, taken throughout the Manx fishing season of 1923, with a view to ascertaining the relation, if any, between age and the chemical composition of the tissues.

The "winter-rings" upon the scales have been taken as a criterion of age, and the samples examined have been homogeneous as regards age and sexual maturity.

At any given stage of sexual maturity, the water-, fat- and protein-content of the fresh muscle-substance are dependent upon age, the older fish containing a lower percentage of water and protein, and a higher percentage of fat.

The variations in composition, with age, resemble closely those associated with the successive stages of sexual maturation, established by Milroy and Johnstone.

Analyses made upon a limited series of herring-samples from the Clyde area, in 1922, indicate the general distribution of water, fat, and protein, between muscle, liver, and gonad, during the middle or "filling" period of maturation. They suggest that the metabolic demands of the growing gonads are met, in the first instance, by the fatty reserves of the liver; the muscular tissue is not depleted until shortly before spawning occurs.

Determinations of phosphorus were made in the case of the Scottish samples. Further work and improved technique will be needed before definite conclusions can be drawn as to the part played by this element, but it is clear that the developing gonads have a high phosphorus-requirement, and that the phosphatides (lecithin, etc.) figure largely in the tissue-changes and translocations associated with maturation.

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LXVII. THE PRODUCTION OF AMMONIA AND UREA IN AUTOLYSIS.

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Since Jacoby [1900] demonstrated an increase of ammonia during autolysis, many workers have studied this question, using varying methods of estimating the NH_3, and obtained very conflicting results, especially on the effect of acid and alkali on the total NH_3 produced, and upon this NH_3 expressed as a percentage of the total uncoagulable nitrogen [Simon, 1910; Bostock, 1912].

The production of urea in liver autolysis, after much contradictory early work, was conclusively demonstrated only by Fosse and Rouchelman [1921], using the xanthydrol technique. They however made no study of the conditions, especially with reference to the NH_3 formed at the same time, and the relation between the two.

In the present work the NH_3 and urea formation in a number of autolysing organs has been studied, and, in some cases, simultaneous determinations of the increase in total uncoagulable and amino N made. The p_H has been controlled throughout.

Methods.

The autolysing digests were made up as follows. 100 g. of the organ, weighed after mincing, were ground fine in a large mortar with or without the addition of sand or ground glass. 25 cc. of toluene were then added, and the mush further ground till a pasty consistency was obtained. 100 cc. of buffer solution were added, the whole thoroughly mixed and transferred to a flask, the residue on the pestle and mortar being washed into the flask with 100 cc. more buffer solution, and well shaken, together with the further addition of 25 cc. toluene. The flask was then corked, and placed in an incubator at 38°. In some cases 50 or even only 25 g. of the organ were used, and the amounts of buffer solution and toluene correspondingly reduced. Using this technique the digests remain perfectly sterile. It is unfortunately necessary to employ some material to prevent bacterial growth, and toluene appears to be the disinfectant which has the least disturbing effect on those processes undergoing investigation, being neutral, insoluble and inert. Samples were withdrawn at once, and again at suitable intervals, coagulated by placing in boiling water, and filtered. A clear yellow filtrate was obtained upon which

the estimations were carried out. This method of sampling is quicker than precipitation with trichloroacetic acid, involves no dilution, and gives satisfactory results.

The buffer solutions used were those of Clark and Lubs, of p_H 2, 3, 4, 5·6 and 7·5. Owing to the buffering action of the tissue proteins and amino acids, the p_H actually obtained with each buffer must be estimated. This was done by the indicator method (using a comparator), on the filtrate on which the estimations were carried out. With the large differences in the series of p_H studied, this method is sufficiently accurate. The p_H of the digests made up in this way did not change appreciably during autolysis, and were as follows:

Table I.

p_H of buffer	p_H of filtrate
2	4·5–4·7
3	5 –5·2
4	5·4–5·6
5·6	5·9–6·1
7·5	6·6–6·8

The method employed for estimating the NH_3 was one devised by Foreman [1920], shortly to be published by him in greater detail and with improved technique. 5 cc. of the filtrate were placed in a steam distillation flask, with 45 cc. 97 % alcohol together with a few drops of phenolphthalein, and soda, approx. *N*, added until the alcoholic solution was faintly pink. Steam distillation was at once commenced into 20 cc. *N*/50 H_2SO_4. Methyl red was used as the indicator in the back-titration of the H_2SO_4 with *N*/50 CO_2-free NaOH. The method is extremely rapid and very accurate.

The urea was estimated by taking 5 cc. of the filtrate, adding 2 cc. urease solution (made by grinding 5 g. soya bean meal with 50 cc. 0·6 % KH_2PO_4, and filtering after 15 minutes), warming for 10 minutes to 45°, and then estimating the total NH_3 present. Estimations of urea by xanthydrol were made in many instances as a check and the two methods agreed very well.

Some of the material used was obtained from animals killed in the laboratory for the purpose (cat, rabbit, hen), but in the main it has been obtained from the slaughter-house immediately after the death of the animal. The effect of cold storage was tried, and found not to influence the results.

Effect of p_H on Ammonia and Urea production.

The curves of Fig. 1 are typical of a number obtained from ox spleen, the increase of NH_3 being shown below and of urea above. The increase of urea is often much greater than shown in this figure. The sharp optimum for both NH_3 and urea at p_H 6 is very marked. The curves obtained from the kidney (ox) are identical in general characters with the above, but differ in the following points:—(1) There is a much smaller increase of NH_3 and urea at every p_H. (2) There is a very much higher initial concentration of urea (from 6–12 times) due to small amounts of formed urine in the tubules. (3) The

increase of urea is confined entirely to the first two days, when it may be more rapid than that of the NH_3. (4) The NH_3 curves do not show such a sharp optimum at p_H 6, and resemble more the NH_3 curves of Fig. 2.

Fig. 2 represents one of seven sets of curves obtained from the liver during the winter. The marked differences from the spleen curves are at once apparent. Whereas the optimum p_H for NH_3 is the same but not so marked,

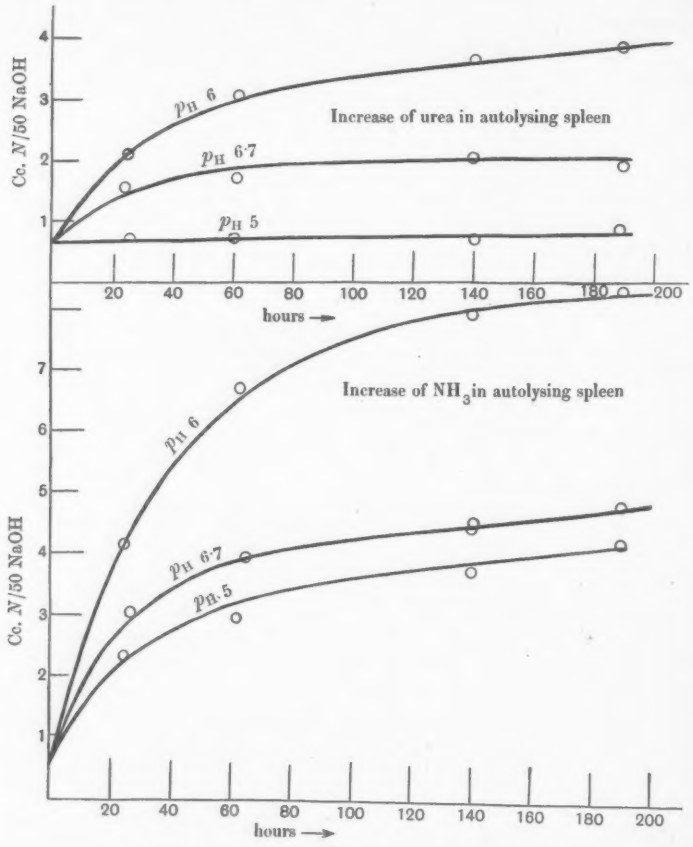

Fig. 1.

there is a greater production of urea on the acid side, at a p_H far from the optimum for NH_3. The urea increase at this p_H is more rapid at first than that of the NH_3, falling off almost to nothing after two days, while the NH_3 increase, slow at first, does not show the same falling off.

During the summer the NH_3 curves are unchanged, but the production of urea at p_H 5–5·4 falls to a value often lower than that at p_H 6. Six sets of

curves were taken in the summer, and no exceptions found, the summer ones all differing from the winter. Attempts to explain these marked differences have so far failed. It is not due to the youth of the animals killed during the summer (calves or lambs), and attempts to reproduce the differences by imitating in laboratory animals (rabbits) the summer and winter feeding of cows and sheep were failures. Seasonal variations in other enzymes [Burge and Leichsenring, 1922] have been described, and may afford the explanation in this case also.

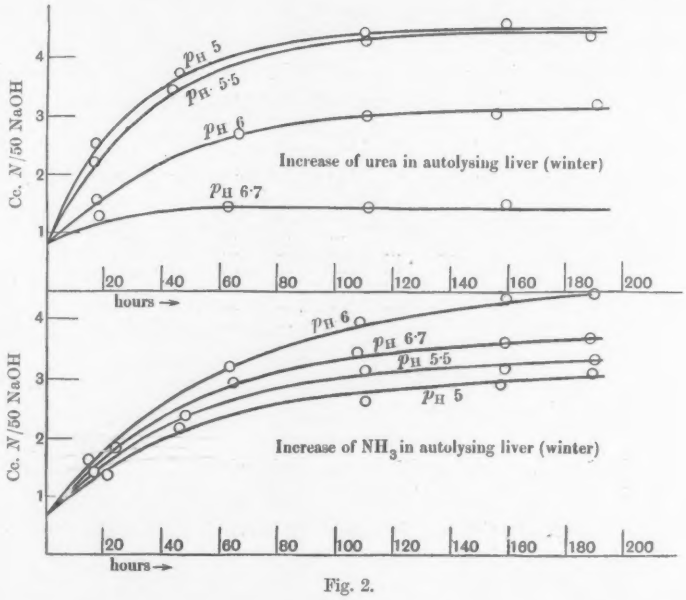

Fig. 2.

Effect of gaseous oxygen, hydrogen or nitrogen on production of Ammonia and Urea.

The effect of these gases on autolysis has been studied by Laqueur [1912], Bellazzi [1908], Morse [1916] and Shima [1922], the first of whom obtained marked inhibition of autolysis by oxygen measured by total soluble and amino N. He used no buffers, and often oxygen under pressure. On the other hand the other three did not obtain this effect, and Morse (estimating total soluble N) certainly took up the work with the idea that oxygen might inhibit autolysis.

Since it seemed that oxygen might increase urea formation by promoting oxidation of amino acids, and possibly other compounds, the effect of oxygen and hydrogen on autolysis, and especially urea formation, was studied in the following way. Two autolysing digests were made up at the same p_H, and,

usually, but not always, evacuated in Buchner flasks or desiccators. Oxygen and hydrogen were then led into each, respectively, from cylinders, after passing through water and toluene. The treatment was continued (with shaking) for about one hour a day for the first three days, and thereafter less frequently. An interesting observation, made repeatedly during these experiments, is the high resistance of reduced haemoglobin to such treatment, its spectrum being visible after several days, whereas oxyhaemoglobin very soon disappears. To ensure the genuineness of the results given below, one experiment was carried out with oxygen made by heating $KClO_3$ with MnO_2, with exactly similar results. A set of these is given below (Table II). Nitrous oxide was also tried, and found not to influence in any way the rate of autolysis, or the effect of oxygen on autolysis. The effect of carbon dioxide is only one of p_H; if this remains constant, this gas has no effect.

Table II. *Spleen Autolysis. Incubation 120 hours at 37°. All determinations were made on the filtrate after boiling and refer to 5 cc. of this filtrate.*

	Oxygen	Hydrogen
Total soluble N in cc. *N*/10 NaOH	27·25	27·25
p_H	5·9	5·9
NH_3 in cc. *N*/50 NaOH	8·4	8·7
Amino acid N, Sörensen titration in cc. *N*/10 NaOH	12·4	12·9
Urea in cc. *N*/50 NaOH	3	5·8
Urea in g. xanthydrol urea compound	0·015 g.	0·028 g.

The results are quite typical. The total soluble N is always the same, and also the p_H. The NH_3 is sometimes the same and sometimes as here a small percentage higher in the hydrogen filtrate. The urea is always very much greater in the hydrogen filtrate, and the amount of urea produced in an autolysis in hydrogen is the same as that in an autolysis with no gas present, so the difference is due only to an inhibition by oxygen, and not to a simultaneous acceleration by hydrogen. Nitrogen and hydrogen give identical results.

Effect of Adrenaline on Urea production.

The results of the experiments with oxygen led to a repetition of some work of Marie [1922], who found that the addition of suitable amounts of adrenaline to an autolysing liver doubled the amount of urea produced, and attempted to relate this to carbohydrate mobilisation and oxidation. Unfortunately his results could not be repeated either with buffered or unbuffered autolysing ox livers (trying several different p_H) or with varying concentrations of adrenaline; and his suggestion that the urea has its origin in carbohydrate oxidation in presence of NH_3 is rendered unlikely also by the experimental finding, that the effect of oxygen is to reduce, not to increase, the production of urea.

Independence of the sources of Urea and NH_3.

It might appear from Fig. 1 that the precursor of the urea formed was the NH_3. That this is not the case, however, is shown by a number of facts

and considerations. (1) If a balance existed between the concentrations of NH_3 and urea, subject to the laws of mass action, one would expect the ratio between them to remain the same throughout autolysis. This is not the case; and it is possible to alter the concentration of NH_3 or urea in the autolysing digest, either by adding the substances themselves, or suitable precursors, without altering in any way the rate of production of the other. (2) In the liver and kidney autolyses, the urea in the early stages increases more rapidly than the NH_3 at some values of p_H, but later more slowly, while the NH_3 curve is smooth and regular throughout. (3) The effect of oxygen, which may greatly reduce the urea production and leave the NH_3 practically untouched, also points to their separate origin. Their production may therefore be considered separately.

Production of Urea.

Unlike the increase of ammonia which is common to all autolyses to a greater or lesser extent, that of urea occurs only in certain organs. It has been found in all the mammalian livers tested (bullock, sheep, pig, rabbit), in all mammalian kidneys (bullock, sheep, pig), and mammalian spleens (bullock, cat). It has not been found in any mammalian pancreas (bullock, pig), lung, skeletal muscle (bullock), or heart muscle (sheep). Nor has it been found in avian liver (hen), which is interesting.

True optimum p_H for Urea Production.

Advantage has been taken of the fact that there is no mechanism for urea formation in the pancreas to study the optimum p_H for urea production, uncomplicated by the disturbing factor of the simultaneous hydrolytic cleavage which is inevitable during autolysis.

450 g. of spleen (ox) were sliced thin, and added slowly to 750 cc. of boiling distilled water. When cool, the tissue was minced and pulped with toluene, as described above, in the same water in which it was boiled, made up to approx. p_H 8 with Na_2CO_3, and 100 cc. pancreatic extract added. Other 100 cc. pancreatic extract were added later, and incubation continued 14 days at 38°, when the whole was made slightly acid, boiled, and filtered. The filtrate was now titrated to p_H 6, using a comparator, and some of the filtrate further titrated to p_H 7. Autolysing spleen digests were made up as described at p_H 6 and 6·8.

A. At p_H 6·8–7 into three boiling tubes:

(1) 20 cc. filtrate from pancreas digest + 0·5 cc. *N* NaOH } + 20 cc. autolysing spleen suspension at p_H 6·8
(2) " " " alone
(3) 20 cc. autolysing spleen suspension at p_H 6·8 alone

B. At p_H 6 into three boiling tubes:

(1) 20 cc. filtrate from pancreas digest + 0·5 cc. H_2O } + 20 cc. autolysing spleen suspension at p_H 6
(2) " " " alone
(3) 20 cc. autolysing spleen suspension at p_H 6 alone

The six tubes were incubated 48 hours at 37°, and then plunged into boiling water. When boiling, (2) and (3) in each case were mixed, and served as the control. After filtering the urea was estimated.

Figures after correction from the controls:

A. Urea formed at p_H 7 from substances in the pancreas-digested spleen, expressed in cc. *N*/50 NaOH per 5 cc. of filtrate ... ... 1·25 cc.
B. The same at p_H 6 ... ... 0·1

An exactly similar experiment on pancreas-digested liver, with liver enzymes, at p_H 5 and 7, gave the following figures:

A. Urea formed at p_H 6·8–7 from substances in the pancreas-digested liver, expressed in cc. *N*/50 NaOH per 5 cc. filtrate ... ... 4·45 cc.
B. The same at p_H 5 ... ... 1·4

A simultaneous autolysis of both spleen and liver at the same p_H showed, as always, a formation of more urea at the more acid p_H. There must be a series of reactions before the urea is set free, the observed optimum p_H (p_H 6) for urea production in autolysis being the reaction at which these several enzymes can best act; and this is quite in accordance with the work of Hedin [1922] and others, the enzyme termed by him "β-protease," and responsible for primary cleavage of the proteins, acting best at a p_H of 4–5, while the formation of urea according to these results is most rapid at p_H 7.

Possible precursors of Urea.

The mammalian, as distinct from the avian, liver is generally agreed to contain large amounts of arginase, which must account for some at least of the urea formed in such organs. Kossel and Dakin [1904] using isolation methods, Edlbacher [1915] and Fuchs [1921] using the Sörensen amino N titration method for estimating arginine, all failed to detect arginase in the mammalian spleen, and this, coupled with the fact that p_H 5 is the optimum for urea formation in the liver in winter, whereas at this p_H no urea whatever is formed in the spleen, points to the source of the urea in the spleen being other than arginine. The true optimum p_H for urea production is however the same in both spleen and liver. Arginase too was undoubtedly present in all the spleens tested in this work, in a concentration so low, that with the methods employed by previous workers it might not easily be detected. Arginase is present also in the kidney. A number of other conceivable precursors have been tried with consistently negative results, *e.g.* nucleic acid, thymine, cytosine, uracil, creatine and creatinine. The theory that the urea has its origin in the oxidation of glucose or amino acids seems untenable. There is evidence however that arginine is not the sole precursor.

The Rôle of Oxygen.

To elucidate this, the effect of oxygen on a pure arginine-arginase system has been most carefully studied. The experiments were carried out in part with the free base arginine obtained from the British Drug Houses, Ltd. (this was 90 % pure) and partly with a supply of arginine nitrate from

F. Hoffmann, La Roche & Cie, S.A., Bâle. The arginase was sometimes prepared by alcohol precipitation from strained liver suspensions. This method gives a dry powder which can be kept, but the alcohol precipitation must be carried out at a low temperature to prevent destruction of the arginase, room temperature being too high in the height of summer. The powder is ground with water and filtered before use. The method of obtaining an arginase preparation described by Edlbacher was also employed. This yields essentially a dilute suspension of the organ in question. The two methods in this case give identical results. The procedure was as follows: 5 cc. of a 0·4 % solution of arginine, 2·1 cc. of a solution of arginase, and eight drops of toluene were placed in each of two test tubes. Oxygen and hydrogen were led slowly through each respectively for 6–24 hours, and they were then transferred to the incubator at 38°. The extent of the action of the arginase was estimated by the urea formed. The results are summarised in Table III, the figures being the urea produced from 5 cc. of the arginine solution expressed in cc. $N/50$ NaOH.

Table III.

Organ	Type of enzyme	Time of treatment with O_2 and H_2 in hours	Time of incubation after passage of gas in hours	p_H	Urea formed	
					Oxygen	Hydrogen
Liver	Alc. ppt.	8	14	6·9–7	5·65	5·65
,,	Dilute suspension	12	24	,,	6	6·1
,,	,,	12	48	,,	6·5	6·5
,,	,,	24	0	,,	5·3	5·4
Spleen	,,	24	14	,,	1·25	1·3
,,	,,	21	14	,,	0·75	0·65
,,	,,	24	14	,,	0·45	0·35
,,	,,	6	24	6·0	1·9	1·8
				and neutralised before incubation		
,,	,,	6	24	6·0 and ditto	0·8	0·7

The table shows clearly that the pure arginine-arginase system is unaffected by a treatment with oxygen, which would undoubtedly suppress to a large extent the formation of urea in an autolysis.

The effect of oxygen is not an irreversible oxidation of the precursor of urea. This is shown by an experiment such as the following. Spleen autolysis in oxygen and hydrogen is carried out as already described, and the urea estimated in the filtrates after heating to 100° (A in Table IV). Dilute liver suspension is now added to each, and incubation continued 14 hours. After heating to 100° the urea is again estimated (B in Table IV). The controls have been deducted.

Table IV.

	A	*B*
Urea in 5 cc. filtrate from autolysis in oxygen	1·9	4·1
,, ,, ,, hydrogen	4·2	4·4

Although not converted into urea, the substrate in an autolysis in oxygen remains untouched, and may be converted into urea by the addition of fresh enzyme.

Autolysis of spleen at p_H 5, digestion of liver or spleen with pepsin and trypsin, autolysis in oxygen, and finally acid hydrolysis of spleen or liver, all give solutions, which when filtered and neutralised, are capable of acting as substrates for the urea-producing enzyme systems of the liver. The effect of oxygen can be shown in this way to be on an enzyme responsible for a part of the urea produced in autolysis.

Liver is ground up as for an autolysis at p_H 6, and strained through muslin into two small filter flasks which are evacuated, treated with the gas for some hours, and incubated at 38° for 12 hours; treated with the gas again for two hours, and incubated 12 hours more. The two suspensions so obtained form the enzyme solutions O and H (Table V) with which the following experiment was performed. The controls have been deducted, and the figures indicate the urea present in 5 cc. samples after 12 hours' incubation.

Table V.

					Urea
20 cc. neutral filtrate of autolysis at p_H 5	+ 2 cc. enzyme	O + 10 drops of toluene			3·95
" " " "	" "	H + 10 "	"		6·65
20 cc. filtrate of autolysis at p_H 5 treated with "pancreatin" 60 hours	" "	O + 10 "	"		5·95
" " " "	" "	H + 10 "	"		7·5
20 cc. filtrate of spleen digested 4 days with pepsin and 10 with trypsin	" "	O + 10 "	"		3·2
" " " "	" "	H + 10 "	"		5·5
20 cc. neutral acid hydrolysis filtrate	" "	O + 10 "	"		(1) 2·2 (2) 4·3 (3) 4·05 (4) 3·6
" " "	" "	H + 10 "	"		(1) 2·1 (2) 4·4 (3) 4·55 (4) 4

This experiment makes clearer the part played by oxygen in autolysis. It would appear also to be chemical not physical, for nitrous oxide which is much more soluble does not produce any effect whatever. The enzyme whose action is suppressed cannot be Hedin's β-protease, or arginase. It is present in spleen, liver and kidney, as the urea production in all three is suppressed by oxygen. It is more active in neutral than acid solution, and appears to be inactivated more or less rapidly at p_H 5. This may account for its action not being apparent at all at this p_H in the spleen; and in the liver, the production of urea at p_H 5 is limited by the inactivation of the enzyme, for after urea production at this p_H has ceased, addition of fresh enzyme leads to further amounts of urea. Arginase [Hunter and Morell, 1922] has an optimum reaction at p_H 7, so that some of the effects obtained on the optimum p_H for urea production in filtrates from trypsin-digested organs must be attributed to this, but it seems that there may be two different enzymes at work simultaneously, and their effects are here additive.

The Nature of the Substrate of this Enzyme.

If this is not arginine in the free state, the other possibilities are (1) that the arginine is present in some combined form on which this enzyme acts, setting free the arginine, to be decomposed in turn by the arginase. (2) That there is some compound, probably so far undescribed, acting as the urea precursor.

If the former is the true interpretation, the compound containing the arginine must be an interesting one. It can hardly be a simple peptide, and the enzyme a kind of ereptase, for the action of the enzyme is so extremely selective, *e.g.* in an autolysis in oxygen the urea may be half the amount of its control in hydrogen, and the ammonia N and amino N practically the same. It is possible that the compound, if it exists, does not retain the arginine moiety by a peptide linkage. At all events it is not decomposed at all readily by trypsin (Table IV), for these digests all gave the bromine reaction for free tryptophan intensely.

The following experiments, however, point to the substrate not containing arginine. They are done on filtrates from spleen autolysis in oxygen and hydrogen, with a preparation of enzymes from a bullock's liver precipitated by alcohol (Fig. 3). Considerably more urea is produced from the filtrates of the autolyses done in oxygen, as already described, and if the concentration of enzyme is low, and incubation not too long, the enzyme becomes the limiting factor in this urea production. The technique is as follows. 5 cc. of the filtrate substrate, and five drops of toluene, are placed

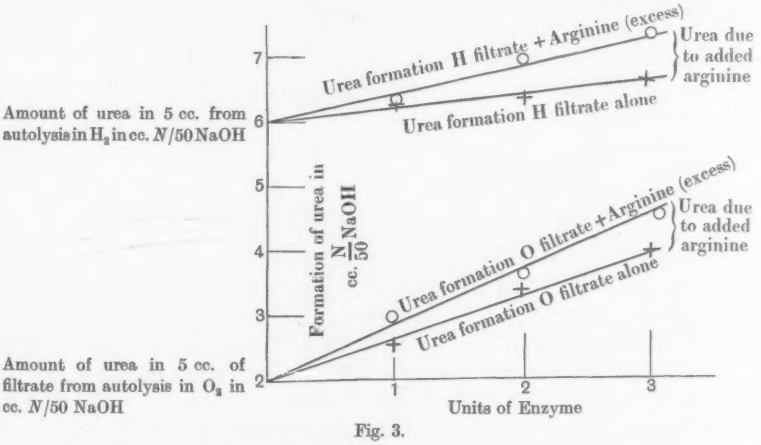
Urea formation H filtrate + Arginine (excess)
Urea due to added arginine
Urea formation H filtrate alone
Amount of urea in 5 cc. from autolysis in H_2 in cc. $N/50$ NaOH
Formation of urea in cc. $\frac{N}{50}$ NaOH
Urea formation O filtrate + Arginine (excess)
Urea due to added arginine
Urea formation O filtrate alone
Amount of urea in 5 cc. of filtrate from autolysis in O_2 in cc. $N/50$ NaOH
Units of Enzyme
7
6
5
4
3
2
1
2
3

Fig. 3.

in each test tube. 1, 2, 3, etc. cc. of enzyme solution are added, as required, and all the tubes made up to a standard volume with distilled water. After incubation they are heated rapidly to 100°, and the urea estimated. The enzyme preparation contains both arginase and the enzyme which is suppressed by oxygen. If all the urea were due ultimately to arginine, on adding excess of arginine to all the tubes, the rate of urea production should now depend solely on the concentration of arginase and on nothing else, and the curves (Fig. 3) of urea production with excess of arginine present would be parallel. This is not the case at all, and the effect of adding arginine is to get an increased production of urea of the same order of magnitude in the filtrates, both from the oxygen and hydrogen experiments, as the figure shows.

It is not quite easy to see why the urea production from the filtrate from the autolysis in hydrogen, though much smaller than that from the filtrate from the autolysis in oxygen, should bear a linear relationship to the enzyme concentration, as it appears, from this and other experiments, to do. Apart from this difficulty the experiment points to the precursor of this urea being other than arginine.

Whatever the compound it appears to be destroyed at any rate almost completely by acid hydrolysis. This is shown by Table IV. The enzyme, which is suppressed by oxygen, appears not to have any substrate upon which to act after 30 hours' acid hydrolysis. Arginine is of course not destroyed by acid hydrolysis. The compound however must exist in a state of combination in the cell and be set free by the action of trypsin. This is shown by the consideration of the observed and the true optimum p_H for autolytic urea production.

The production of Ammonia and its possible Precursors.

The amount of ammonia produced varies greatly from one organ to another, being much greater in glandular than in muscular tissues. Earlier workers claimed that its source was to some extent amino acids, though it is now recognised generally to be acid amides present in the protein molecule, and to a small extent the purines of nucleic acid [Jones, 1920]. Glycine, and free cytosine, uracil and thymine have been tried in this work and suffer no deamination.

Optimum p_H for Ammonia production.

This from Figs. 1 and 2 is p_H 6, but this is not the optimum for general autolysis, as measured by total soluble N or amino N in either organ [Dernby, 1918; Bradley, 1923]. p_H 6 is evidently the observed optimum, because at this p_H, as in the case of urea, two or more enzyme systems can act best either independently or consecutively. This is supported by studying the production of ammonia by spleen enzymes from a pancreas-digested spleen, at different p_H, exactly as described above for urea (Table VI).

Table VI.

NH_3 formed from trypsin-digested spleen expressed in cc. $N/50$ cc. soda per 5 cc. of filtrate at p_H 6·8–7	2·45
The same at p_H 6·0	0·1

In this connection treatment of asparagine by tissues is interesting, although asparagine may not be a normal tissue constituent. It is deamidised by both liver [Clementi, 1922] and spleen, and the rate of deamidisation falls off rapidly on the acid side. The following figures have been obtained with liver showing the rates of deamidisation at different p_H. The experiments were done with a 1 % solution of asparagine and a dilute ox-liver suspension.

Table VII.

p_H	NH_3 produced in cc. $N/50$ NaOH per 5 cc.
5	0·2
6	3·3
6·9	8·2

The primary proteolytic enzyme system acting best at more acid reactions than p_H 6, can only deamidise some of the substances present in such an organ as the spleen, and capable of yielding ammonia under the action of its own tissue enzymes. The remainder are only deamidised by an enzyme system acting best at p_H 7. This enzyme system however is not capable of hydrolysing the tissue proteins. The reaction at which these two systems can best combine is p_H 6. At a more acid reaction than this, though hydrolysis is active, the deamidising enzyme system, acting best at p_H 7, is inactive; on the alkaline side of p_H 6 the activities of this system are limited by the small amount of hydrolysis and consequently of substrate.

These experiments show the source of the conflicting results obtained by early workers on the absolute ammonia production, and the same production expressed as a percentage of the total soluble N.

Summary.

(1) The effect of oxygen, hydrogen, nitrous oxide and adrenaline, and of p_H on the formation of NH_3 and urea in autolysis has been investigated.

(2) Ammonia and urea have been shown to be formed by separate processes, and their precursors have been discussed.

(3) An enzyme which forms part of the urea-producing system has been described, which is strongly inhibited by gaseous oxygen. Its substrate has been considered.

(4) This enzyme system is under further investigation.

The author wishes to express his gratitude to Profs. Peters and Hopkins for their valuable advice and criticism.

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LXVIII. THE EFFECT OF OXYGEN SUPPLY ON THE METABOLISM OF *BACILLUS COLI COMMUNIS*.

By MARJORY STEPHENSON
and MARGARET DAMPIER WHETHAM.

A report to the Medical Research Council.

From the Biochemical Laboratory, Cambridge.

(*Received April 1st, 1924.*)

In work already published [Stephenson and Whetham, 1923], the respiratory exchange of a growing culture of the Timothy Grass Bacillus was studied, firstly during a period of growth on glucose and secondly during a period of starvation after the glucose had been used up. This organism is characteristically non-fermenting; that is, when grown on a synthetic medium with glucose as the sole source of carbon, and ammonia as the source of nitrogen, no intermediate products collect in the medium in any measurable quantity. Moreover, the carbon of the glucose can all be accounted for in the carbon dioxide given off by the organism, on the one hand, and in the carbon of the organism itself, on the other, with the exception of small traces of carbonaceous material in the medium, which are attributable to the autolytic products of dead bacteria.

Comparison with a fermenting organism seemed desirable, *i.e.* one which breaks down carbohydrates and other compounds in such a way that intermediate products collect in the medium. As an example of such a type of organism *Bacillus coli communis* was chosen.

The apparatus used consisted of four sections, *A*, a respiration flask, kept in a water-bath at 37°, *B* and *C*, sulphuric acid and pumice absorption tubes, *D*, potash bulbs. Dry air, free from carbon dioxide, was passed continuously through the apparatus, which was cooled and weighed at intervals. The total gain in weight over any period gave the oxygen uptake, while the gain in weight of *C* and *D* gave the carbon dioxide output. The technique is fully described in our previous communication [1923]. The application of this method to the study of a fermenting organism presents a difficulty in the possible carrying over of volatile products from the respiration flask to the absorption tubes or beyond. The products of the action of *B. coli* on glucose have been examined by a number of investigators. Grey [1914, 1917] in particular has made a careful quantitative study and has shown the presence

of lactic, succinic, acetic and formic acids, ethyl alcohol, and hydrogen. Blank experiments were accordingly made by us with formic and acetic acids and with alcohol, in concentrations exceeding anything found by Grey. No measurable amounts of these substances were carried over.

The question of hydrogen, however, remained unsettled. It will be apparent that any hydrogen produced in the respiration flask would be carried out through the absorption system and be lost, causing a loss of weight which would invalidate the figures for oxygen uptake. From Grey's results we learn that hydrogen is produced from glucose by *B. coli* in quantities not exceeding 0·5 % of the weight of glucose fermented. The total amount of glucose used by us in each experiment was 0·5 g., and any loss from hydrogen would thus probably not exceed 0·0025 g., an amount lying within our experimental error (± 0·004 g. on each weighing of the whole system). Nevertheless it appeared prudent to investigate this point. The escaping gases were passed over a red-hot platinum spiral and then through a calcium chloride absorption tube, the hydrogen being thus estimated by weight as water. The apparatus was blanked with known quantities of hydrogen and found satisfactory. In one experiment, 0·0145 g. water was produced, corresponding to 0·0016 g. of hydrogen, from 0·5 g. of glucose. Since such an amount falls within the experimental error and therefore does not affect the figures for oxygen uptake, any production of hydrogen in our experiments was neglected.

An inorganic medium of the following composition was used:

$(NH_4)_2HPO_4$ ... 0·4 %; KH_2PO_4 ... 0·1 %; $MgSO_4 . 7H_2O$ ... 0·07 %;

the hydrogen ion concentration being about p_H 7·6. 50 cc. of medium were introduced into the sterile respiration flask, together with 0·5 g. of the carbon compound under investigation. Organic acids were added in the form of the ammonium salt. The medium was sown with a loopful of a 24 hour broth culture of *B. coli*. Three strains of *B. coli* were used in the course of the experiments, and were found to give similar results. Subcultures were made and examined after each experiment to ensure that the culture had remained free from contamination.

Experiments on glucose.

Fig. 1 shows the oxygen uptake and carbon dioxide output of *B. coli* on 1 % glucose inorganic medium, bubbled continuously with air. At the end of 24 hours, though extensive growth had occurred and the medium had already become acid, no oxygen had been taken up and the carbon dioxide output was very low. Subsequently a small oxygen uptake is recorded. Fig. 2 shows the results of an identical experiment made at a different time with a different strain of *B. coli*. It will be recalled that *B. coli* is a facultative anaerobe. It would seem that in the early stages of glucose breakdown, *B. coli* behaves as an anaerobe even in the presence of air.

In an atmosphere of oxygen, on the other hand, the type of metabolism of the organism is changed. Fig. 3 shows the greatly increased oxygen uptake and carbon dioxide output of *B. coli* on a glucose medium bubbled with oxygen instead of with air. The oxygen was displaced by air before each weighing to avoid the errors consequent on the difference of density between air and oxygen. If this precaution is omitted, an error amounting to 0·03 g. may be introduced. Blank experiments prove that at least two litres of air must be passed through the system in order satisfactorily to displace the oxygen.

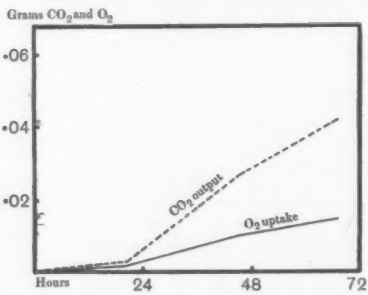

Fig. 1. Carbon dioxide output and oxygen uptake of *B. coli* on 1 % glucose in air.

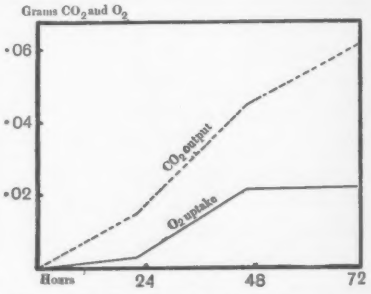

Fig. 2. Carbon dioxide output and oxygen uptake of *B. coli* on 1 % glucose in air.

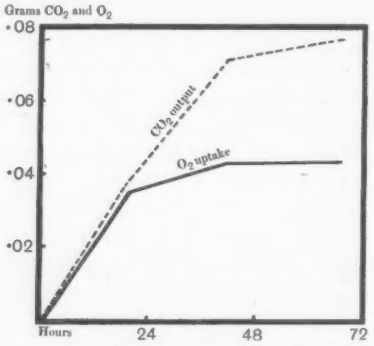

Fig. 3. Carbon dioxide output and oxygen uptake of *B. coli* on 1 % glucose in oxygen.

The increased gaseous exchange of *B. coli* in oxygen might at first be ascribed to a speeding up of the entire metabolism of the cell. This, however, is not the whole truth. The oxygen uptake is proportionately more increased than the carbon dioxide output; moreover, if the effect of the increased pressure of oxygen were merely to push up the metabolism as a whole, it would seem likely that the carbon dioxide output and consumption of glucose would be increased by the same ratio. This is not the case. In a pair of

parallel experiments, each supplied with 0·53 g. of glucose, the organism in oxygen broke down 0·295 g. glucose and gave off 0·0598 g. carbon dioxide, while that in air utilised nearly as much glucose, namely 0·28 g., but only evolved 0·0350 g. carbon dioxide. Furthermore, in an experiment in which the disappearance of glucose was followed in air and oxygen respectively, the amount of glucose broken down in the two cases was found to be identical (Fig. 4). The longer latent period in oxygen which is apparent in Fig. 4 was a fairly constant phenomenon with experiments on glucose inorganic medium, and may perhaps be attributed to a higher mortality among the bacteria originally sown, due to the more drastic change in environment. Experiments in which glucose was added to tryptic broth [Cole and Onslow, 1916], instead of to inorganic medium, gave results closely parallel with those described above except in this one respect: with broth, there was no difference in length of latent period between cultures kept in air and those kept in oxygen.

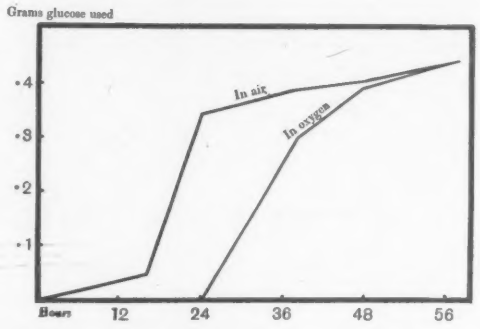

Fig. 4. Utilisation of glucose by *B. coli* in air and in oxygen.

Verzár, Nábráczky and Szányi [1923] have shown that *B. coli* ceases to break down glucose at a p_H of 4·08. If the medium is heavily buffered, more acid accumulates before this limiting p_H is reached, and if the glucose is not too concentrated, the medium sufficiently buffered, and of a sufficiently high p_H to begin with, the whole of the glucose may be utilised. In our experiments with inorganic medium, the limiting p_H was reached when still about half the glucose was left. When broth was substituted for inorganic medium, the increase of buffer substances enabled more glucose to be utilised. The effect of oxygen now became clearer (see Fig. 5). The carbon dioxide output was not only greater in oxygen than in air, but continued for a longer time. It seemed probable, therefore, that in an atmosphere of oxygen the organism was able to remove one of the acid breakdown products of glucose to a greater extent than in air, thus deferring the appearance of the limiting p_H. In the analyses of Grey, lactic acid was the chief product of the breakdown of glucose

by *B. coli*, and we consequently studied the oxygen uptake and carbon dioxide output of *B. coli* on a medium containing 1·0 % lactic acid (as ammonium lactate) as the sole source of carbon.

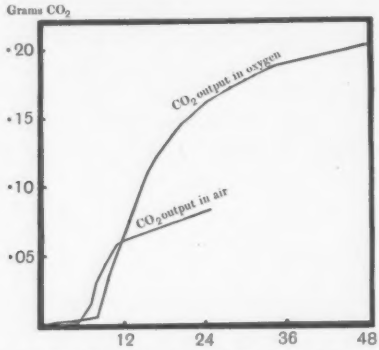

Fig. 5. Carbon dioxide output of *B. coli* on 1 % glucose broth in oxygen and in air.

Experiments on lactic acid.

The inorganic medium used was the one described above. The lactic acid was estimated at the beginning and end of the experiment by the micromethod of Meyerhof [1920, 1921]. In order to test the validity of this method for a fermentation liquid containing a variety of products, a mixture of the following composition was made up in inorganic medium: ethyl alcohol, 1 %; lactic, acetic and formic acids, each 1 %; succinic acid, 0·16 %. 50 cc. of this were

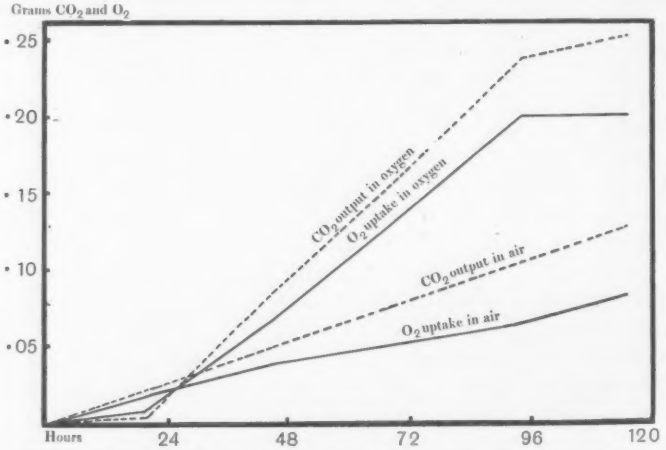

Fig. 6. Carbon dioxide output and oxygen uptake of *B. coli* on ammonium lactate in oxygen and in air.
[Note: some CO_2 fixed in the medium.]

acidified with phosphoric acid and the volatile products distilled off *in vacuo* at 60° till about 10 cc. remained in the distilling flask: this was washed out, made up to 1 litre, and 10 cc. taken for the estimation. It has been shown by many workers that succinic acid is not oxidised by permanganate under the conditions of the Meyerhof method; our experiments confirm this. For example, in one estimation, 0·0051 g. of lactic acid was present in 10 cc. of diluted solution: 0·0048 g. and 0·0049 g. were found by two estimations. For the estimation of the lactic acid remaining in the respiration flask after an experiment, the organism was removed by centrifuging the liquid after the volatile products had been distilled off and the volume of the residue made up to 500 cc.

The figures for the respiratory exchange of *B. coli* on lactic acid in oxygen and in air are recorded in Fig. 6. The medium in this case becomes more alkaline as growth proceeds, owing to the liberation of ammonia. A certain amount of carbon dioxide remains in the medium as ammonium carbonate: this was driven into the potash bulbs at the end of each experiment by aeration after the addition of about 2 cc. of syrupy phosphoric acid to the contents of the respiration flask. The amount found was 0·0362 g. in oxygen, and 0·0137 g. in air. Increase of oxygen pressure is shown to increase both oxygen uptake and carbon dioxide output on a lactic acid medium, and at the same time, the breakdown of lactic acid is also increased.

Lactic acid present at beginning of experiment ...	0·41 g.	
Lactic acid present at end of experiment in air ...	0·176 g.	used 0·234 g.
Lactic acid present at end of experiment in oxygen	0·00 g.	used 0·41 g.

The amount of growth in the two cases was compared. The contents of the respiration flask were made up to 100 cc. and centrifuged, the clear liquid was poured off and the bacteria mixed afresh with water and again centrifuged; this was repeated and the washed bacteria were transferred to a platinum dish. The water was evaporated off and the dish dried to constant weight at 105°, and weighed. The dish was then heated to redness, cooled and again weighed, the weight of combustible material (bacterial growth) was thus obtained and found to amount to 0·0258 g. in air and 0·0465 g. in oxygen.

In view of the results obtained in air and in oxygen, a sowing of *B. coli* on ammonium lactate was kept under anaerobic conditions by passing a stream of nitrogen through the respiration flask. A tower containing standard baryta solution was employed instead of potash bulbs to absorb carbon dioxide, enabling the carbon dioxide output to be followed without weighing, and making possible the determination of small amounts at frequent intervals. No growth whatever occurred, and no carbon dioxide was evolved on any occasion from an ammonium lactate medium in nitrogen. But if such a medium, after three or four days in nitrogen, was bubbled with air or oxygen, growth of the organism took place after a short latent period, and carbon dioxide was rapidly evolved. Such an experiment is shown graphically in Fig. 7.

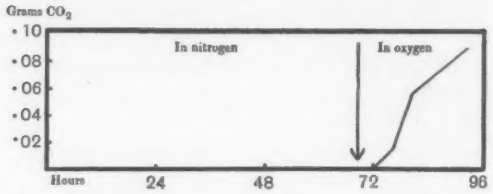

Fig. 7. Carbon dioxide output of *B. coli* on ammonium lactate, first in nitrogen, then in oxygen.

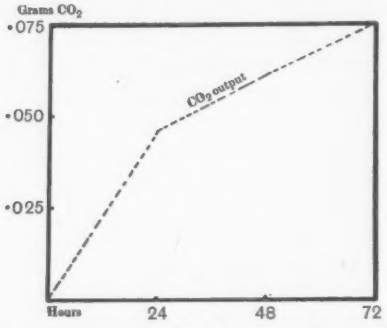

Fig. 8. Carbon dioxide output of *B. coli* on 1 % glucose in nitrogen.

Anaerobic growth of *B. coli* on glucose is of course well known to occur. There is rapid production of carbon dioxide, falling off in about three days owing to accumulation of acid (see Fig. 8). Experiments were made with other known products of glucose as well as with lactic acid, and the observations are summarised below. The carbon dioxide was in each case estimated by baryta titrations. Absence of growth and carbon dioxide output were not regarded as certain until the experiment had lasted about five days. When no growth took place in air, the effect of oxygen was tried.

Growth of B. coli on various sources of carbon.

Inorganic medium with 1 %	Growth in nitrogen	Growth in air	Growth in oxygen
Glucose	+	+	+
Ammonium lactate	−	+	+
,, succinate	−	+	·
,, acetate	−	+	·
Glycerol	−	+	·
Ethyl alcohol	−	−	−

Observations of a similar kind have been made by Braun and Cahn-Bronner [1921], who found that *B. coli* would not grow anaerobically on a lactate medium, and by Fürth and Lieben [1922] who showed that the rate of utilisation of lactic acid by yeast was increased by bubbling the medium with oxygen.

An explanation of these results may be sought in the energy relations of

the reactions involved. If an organism is to grow anaerobically on a simple carbon compound, this compound must be able to supply it not only with the material but also with the energy for growth. The first stage in the breakdown of the compound must not involve the interposition of oxygen and must proceed with evolution of energy. Glucose can be broken down anaerobically into (say) two molecules of lactic acid with the evolution of 22·56 Calories per molecule of glucose. We might consequently expect anaerobic growth on glucose, and in fact we find it to occur. With lactic acid, on the other hand, all the likely paths of anaerobic breakdown appear to involve an absorption of energy. For example, calculating from the heats of combustion of the compounds (as recorded by Landolt and Börnstein [1923]) we have

$CH_3 . CHOH . COOH$[1] $\rightarrow CH_3 . CH_2 . COOH + O$ $-41{\cdot}58$ Cal.
$CH_3 . CHOH . COOH \rightarrow CH_3 . CO . COOH + H_2$ $-$(about) 18 Cal.
$CH_3 . CHOH . COOH \rightarrow CH_2 : CH . COOH + H_2O$ $-2{\cdot}2$ Cal.
$CH_3 . CHOH . COOH \rightarrow CH_3 . CH_2OH + CO_2$ $-14{\cdot}5$ Cal.
$CH_3 . CHOH . COOH \rightarrow CH_3 . CHO + CO_2 +$ $-H_2$ $24{\cdot}6$ Cal. etc.

The same is true of succinic acid:

$$\begin{array}{l} CH_2 . COOH \\ | \\ CH_2 . COOH \end{array} \rightarrow \begin{array}{l} CH . COOH \\ \| \\ CH . COOH \end{array} + H_2 \qquad -31{\cdot}7 \text{ Cal.}$$

$$\begin{array}{l} CH_2 . COOH \\ | \\ CH_2 . COOH \end{array} \rightarrow \begin{array}{l} CH_3 \\ | \\ CH_2 . COOH \end{array} + CO_2 \qquad -10{\cdot}4 \text{ Cal.}$$

etc.

In the case of glycerol, the heats of combustion of likely breakdown products such as hydroxyacetone and lactic aldehyde are not known: deducing them from those of compounds of similar constitution, all the reactions appear to involve absorption of energy.

From such considerations it would appear that energy cannot be obtained from the anaerobic breakdown of lactic and succinic acids and glycerol, and hence it is unlikely that any of them will serve as sole source of carbon to an organism growing anaerobically. The inability of *B. coli* to grow anaerobically on these substances may perhaps thus be explained. We wish to offer this hypothesis in a tentative manner only, but hope later to investigate the matter further in collaboration with Mr J. H. Quastel, who was led to consider the subject from this aspect by his work on *B. pyocyaneus*.

When *B. coli* and other fermenting organisms attack carbohydrates, they liberate acid products. In anaerobic conditions, these products accumulate, since the organism is probably only able to utilise them to a small extent by employing part of the energy obtained from the initial exothermic breakdown of carbohydrate. When oxygen is supplied at atmospheric pressure, the acids are slowly attacked, and poisoning of the cell by acid is delayed. When the concentration of oxygen is increased, the oxidation of the acids is hastened and the metabolism approaches more nearly that of the non-fermenting type of organism. We are reminded of Pasteur's dictum, "La fermentation est la vie sans air."

[1] The heat of combustion of lactic acid was taken at 325·72 Cal. as recently re-determined by Meyerhof [1922]

SUMMARY.

1. A technique previously applied to the study of the metabolism of a non-fermenting bacillus was extended to one of a fermenting type, *B. coli* being employed.

2. The carbon dioxide output and oxygen uptake of *B. coli* on a glucose inorganic medium were followed. The organism was found to be almost anaerobic during the early stages of glucose breakdown.

3. The effect of an atmosphere of oxygen was observed. There was an increase in carbon dioxide output and a larger increase in oxygen uptake.

4. This did not correspond, in the early stages of the experiment, to an increased utilisation of glucose. Metabolism continued longer in oxygen than in air, the limiting p_H not being reached so soon. The increase of gaseous exchange with increase of oxygen pressure was therefore attributed to an effect on some acid breakdown product of glucose.

5. Growth on lactic acid (as the ammonium salt) was studied in air and in oxygen. The disappearance of lactic acid and production of carbon dioxide were greatly increased with increased oxygen tension.

6. Growth did not occur on lactic acid in anaerobic conditions.

7. Succinic and acetic acids and glycerol were also found to be suitable sources of carbon for *B. coli* in air but not in anaerobic conditions.

8. A possible explanation of these facts may be found in the energy relations of the reactions involved.

The authors wish to thank Prof. F. G. Hopkins for his criticism, advice, and encouragement, throughout this work.

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LXIX. THE OXYGEN UPTAKE OF BACTERIA.

By ANNE BARBARA CALLOW.

Report to the Medical Research Council.

From the Biochemical Laboratory, Cambridge.

(*Received April 6th, 1924.*)

THERE appears to be no reference in the literature to the basal metabolism of bacteria, that is the respiration of bacteria which have been washed free from culture medium and are therefore unable to grow, but are able to oxidise the material of their own cells. Differences in the oxygen uptake of different types of bacteria would indicate differences in constitution of the cells. In the present investigation, the oxygen uptake of a number of bacteria, aerobic and anaerobic, has been measured, and it appears to be fairly constant for each strain, but reveals many striking points of difference in different species of bacteria. Interesting results have also been obtained by using bacteria which have been dried in a vacuum desiccator over sulphuric acid. The dried bacteria readily yield a fine powder which can be weighed out, emulsified in buffer solution and examined for oxygen uptake. Judging by the work of Shattock and Dudgeon [1912] it is unlikely that bacteria are killed by the rapid drying process used in these experiments. Throughout the paper when the oxygen uptake of "dried" bacteria is mentioned, it must be understood that the dried powder is suspended in buffer solution.

METHOD.

The apparatus used was Barcroft's micro-respirometer with flat-bottomed bottles, specially designed for the respiration of tissue. Caustic soda was placed in the small cups to absorb CO_2 and the oxygen uptake at 22° was measured.

Bacteria were grown on agar plates or in Roux bottles at 37° for 18 hours, except in the case of slowly growing organisms which were grown for two or three days. The medium was tryptic agar or tryptic broth, with or without the addition of 1% glucose.

Broth cultures were centrifugalised, the clear liquid removed, and the bacteria washed twice with distilled water to remove traces of broth, the final deposit being emulsified in water or buffer and used for the determinations. In the case of agar cultures, the growth was carefully removed from the surface of the agar and washed twice with distilled water.

Cultures of the anaerobe *B. sporogenes* had to be treated in a special way because the large amount of gas (H_2, H_2S, etc.) produced during growth was not removed by washing, and interfered with the measurement of oxygen uptake. The following technique was adopted to avoid this difficulty. The washed bacteria, suspended in distilled water, were cooled in ice for an hour or two, during which time a stream of sterile nitrogen was passed through. The suspension was then centrifugalised, washed with distilled water and used for the Barcroft experiment.

For experiments on dried bacteria, the washed deposit was placed in a small dish and dried in a vacuum desiccator over sulphuric acid for 18 hours. The resulting dried mass was powdered finely and 0·1 g. was weighed out for each experiment. Before drying, it is more difficult to take a known weight, as it is unsatisfactory to weigh the washed bacteria. The following procedure, which gives the approximate weight (expressed as dry weight), was therefore used throughout. The washed bacteria were carefully emulsified in 8 cc. buffer solution, 3 cc. being placed in the Barcroft bottle, and an equivalent 3 cc. in a tared centrifuge tube. The latter was centrifugalised, the buffer solution poured away, the deposit washed with distilled water and again centrifugalised. The water was decanted off and the tube dried in a vacuum desiccator and weighed, thus giving the approximate dry weight of the bacteria placed in the Barcroft bottle. All results were calculated as cc. of oxygen per g. of dried bacteria.

Effect of hydrogen ion concentration of the buffer solution.

Since the hydrogen ion concentration is of great importance in certain oxidations it was considered advisable to find the oxygen uptake of bacteria in buffers of different p_H. Sterile buffer solutions [p_H 6·6, 7·6 and 8·6] were prepared from a basal solution containing 1 % KH_2PO_4. *B. pyocyaneus* was grown for 18 hours in three Roux bottles of broth, then centrifugalised and washed twice with distilled water. The deposit was divided into three portions:

1. Emulsified in 8 cc. buffer of p_H 6·6, 3 cc. being taken for the Barcroft bottle and 3 cc. for the weight determination.
2. Emulsified in buffer of p_H 7·6.
3. Emulsified in buffer of p_H 8·6.

The results of the Barcroft experiments were then calculated as cc. per g. (dry weight). Fig. 1 shows that the three experiments show remarkable agreement, considering that in each case the weight of bacteria used was determined separately.

This experiment was repeated with dried *B. pyocyaneus*, also with *B. prodigiosus* and *B. proteus* with similar results, thus showing that changes in p_H between 6·6 and 8·6 have practically no effect on the oxygen uptake of these bacteria.

The effect of replacing buffer solution by distilled water.

This effect was investigated in order to see whether the phosphate buffer either accelerated or inhibited oxygen uptake.

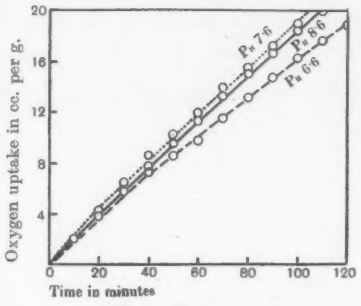

Fig. 1. *B. pyocyaneus*

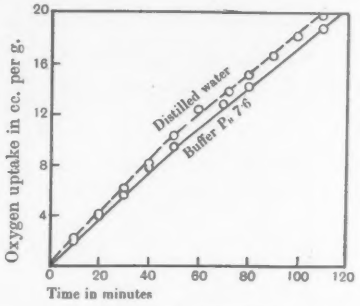

Fig. 2. *B. pyocyaneus.*

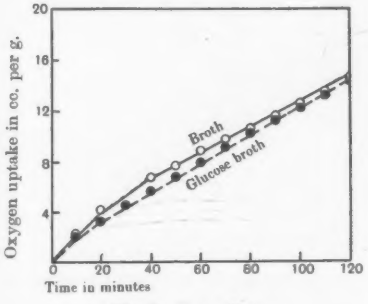

Fig. 3. *B. prodigiosus*

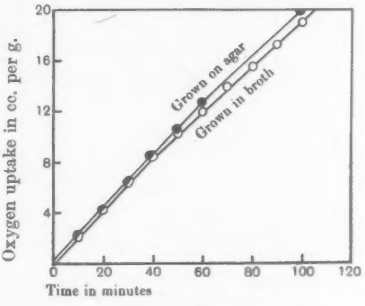

Fig. 4. *B. pyocyaneus.*

Fig. 2 shows that the oxygen uptake of *B. pyocyaneus* in sterile 1 % phosphate buffer at p_H 7·6 is identical with that in sterile distilled water. This experiment has been repeated with *B. alcaligenes*, *B. sporogenes* and *Streptococcus acidi lactici*, and in each case the oxygen uptake was practically the same in buffer as in distilled water. These three organisms were also dried and emulsified in Clark and Lubs' phosphate buffer (sterile) and borate buffer, and here again the results were almost identical with those obtained with distilled water.

For all subsequent experiments a sterile 1 % phosphate buffer, p_H 7·6, was used.

The effect of growing bacteria on different media.

As some of the bacteria examined grew better in glucose broth than in plain broth, it was important to see whether this change in the medium

affected the oxygen uptake. *B. prodigiosus* was therefore grown for 18 hours in broth and in glucose broth, and Fig. 3 shows that the oxygen uptake was the same in each case. Fig. 4 shows the same similarity in oxygen uptake in the case of *B. pyocyaneus* when grown on agar and in broth.

The effect of autoclaving bacteria.

After being autoclaved at 120° for 15 minutes, *B. alcaligenes* and *S. acidi lactici* were unable to take up any oxygen, in an experiment lasting three hours.

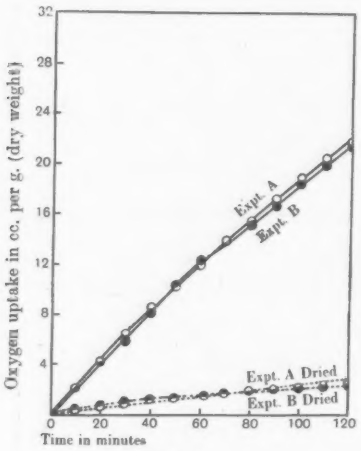

Fig. 5.
B. pyocyaneus. Duplicate Experiments.

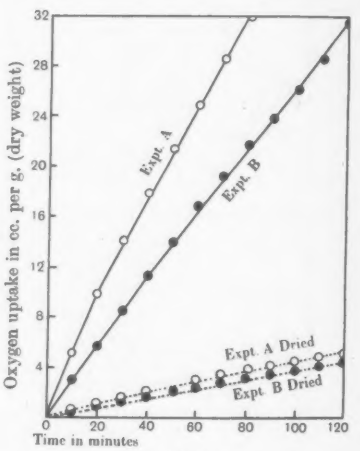

Fig. 6.
B. fluorescens. Duplicate Experiments.

THE OXYGEN UPTAKE OF AEROBIC AND ANAEROBIC BACTERIA.

The following experiments deal with the oxygen uptake of 13 species of bacteria, 12 aerobes and 1 anaerobe, both before and after having been dried *in vacuo*.

The results of duplicate experiments (*A* and *B*) are given graphically so that the amount of variation in each organism can be seen. Cultures were grown in tryptic broth for 18 hours at 37° unless otherwise stated. Bacteria were in every case washed twice with distilled water and emulsified finally in sterile buffer, p_H 7·6.

I. *Aerobic Bacilli without spores.*

1. *B. pyocyaneus.* Fig. 5 shows that the results of the duplicate experiments *A* and *B* are almost identical. In two hours, *B. pyocyaneus* emulsified in buffer takes up 22 cc. of oxygen per g. of dry weight, but, when the bacillus

has been dried and then again emulsified in buffer, the uptake is reduced to 2 cc. Both before and after drying, the oxygen uptake is quite regular, and can be represented graphically by straight lines. Subsequent experiments show that this linear uptake is characteristic of most aerobic bacteria.

2. *B. fluorescens.* The oxygen uptake of *B. fluorescens* (see Fig. 6), like that of *B. pyocyaneus*, is much reduced when the organism is dried, and here

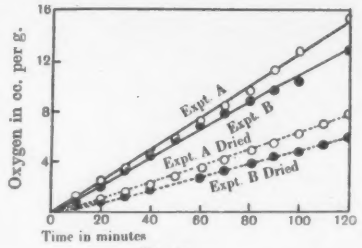

Fig. 7.
B. prodigiosus. Duplicate experiments.

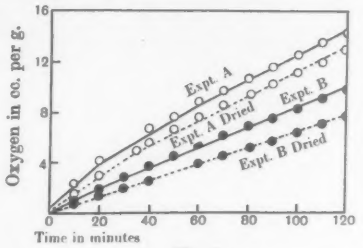

Fig. 8.
B. alcaligenes. Duplicate experiments.

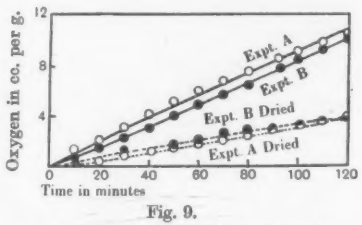

Fig. 9.
B. proteus. Duplicate experiments.

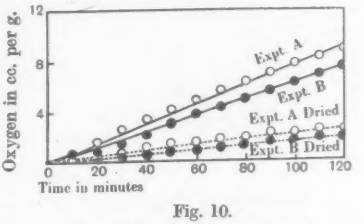

Fig. 10.
B. coli. Duplicate experiments.

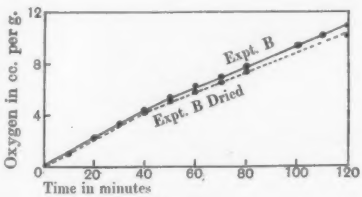

Fig. 11.
Timothy Grass Bacillus

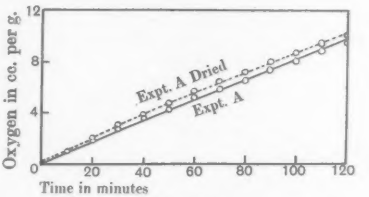

Fig. 12.
Timothy Grass Bacillus

again the rate of uptake shows no falling off either in the normal or dried bacteria.

3. *B. prodigiosus* (Fig. 7) shows a certain amount of reduction in the oxygen uptake in the dried bacteria, but not as much as do *B. pyocyaneus* and *B. fluorescens.*

4. *B. alcaligenes* (Fig. 8) is remarkable because it takes up almost as much oxygen after drying as before.

5. *B. proteus* (Fig. 9). 6. *B. coli* (Fig. 10). These two organisms resemble *B. prodigiosus* in that they are somewhat affected by drying.

7. *Timothy Grass Bacillus* was grown for three days in glucose broth in order to obtain good growth. The results obtained (Figs. 11 and 12) are of special interest as the process of drying has no effect on the oxygen uptake. This is probably due to the protective action of the fatty envelope of this acid-fast organism.

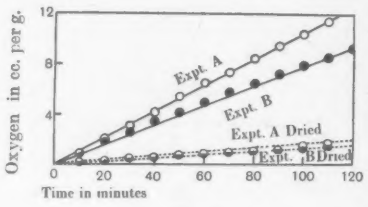

Fig. 13.
B. megatherium. Duplicate experiments.

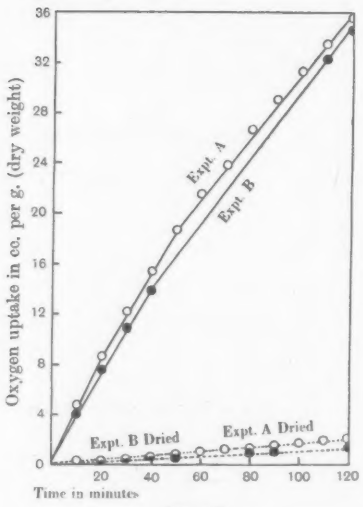

Fig. 14.
B. subtilis. Duplicate experiments.

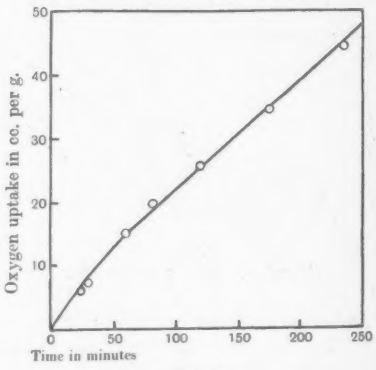

Fig. 15.
B. subtilis.

II. *Aerobic spore-bearing Bacilli.*

8. *B. megatherium* was grown for 18 hours at 37°. The culture consisted almost entirely of bacilli without spores. Fig. 13 shows that with this organism there is a considerable reduction of oxygen uptake after drying.

9. *B. subtilis* was grown for 18 hours at 37°, and the culture consisted almost entirely of bacilli without spores. Fig. 14 shows the very large uptake of *B. subtilis*, which is a strict aerobe, incapable of growing anaerobically. On drying, the oxygen uptake is greatly reduced, as with *B. pyocyaneus*. An older sporing culture gave much lower results: 7·5 cc. per g. in two hours, and 1·0 cc. per g. in two hours after being dried. However, it is impossible to say that this reduction was entirely due to spore formation as, in order to obtain a culture composed chiefly of spores, growth was continued for four

days, and other experiments have shown that four day cultures of non-sporing bacilli take up less oxygen than 18 hour cultures of the same organism. For example, a culture of *B. alcaligenes*, which took up 11 cc. oxygen per g. in two hours when 18 hours old, only took up 7 cc. in the same time when four days old. The same culture dried took up 8 cc. when 18 hours old and 4 cc. when four days old.

Fig. 15 shows the oxygen uptake of an 18 hour culture of *B. subtilis* continued over a period of six hours, during which time the rate of uptake remains quite regular.

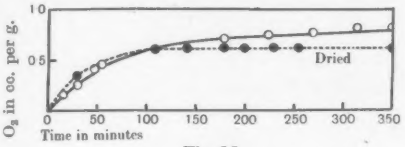

Fig. 16.
B. sporogenes Metchnikoff.

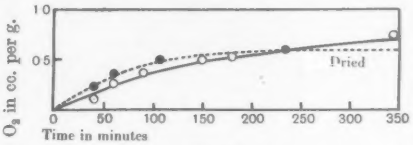

Fig. 20.
Streptococcus acidi lactici.

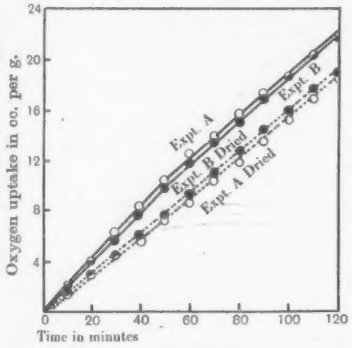

Fig. 17.
Staphylococcus aureus. Duplicate experiments.

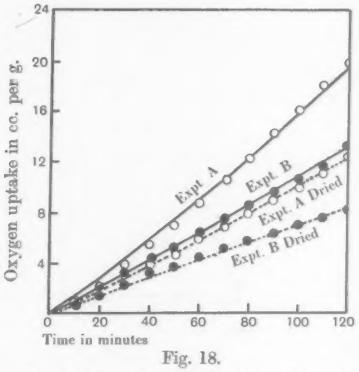

Fig. 18.
Sarcina aurantiaca. Duplicate experiments.

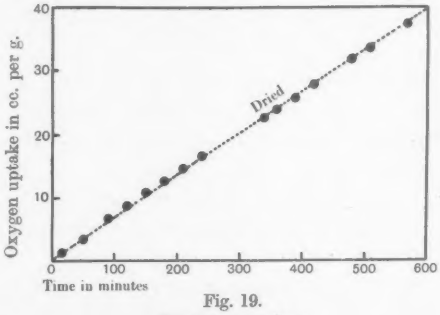

Fig. 19.
Sarcina aurantiaca.

III. *Anaerobic spore-bearing Bacillus.*

10. *B. sporogenes* was grown anaerobically for two days in glucose broth, about 3 litres of medium being required for each experiment. The culture consisted almost entirely of bacilli without spores. Both before and after drying the oxygen uptake of *B. sporogenes* is so minute that the graph had to be drawn to a much larger scale than has been used for all the previous work (Fig. 16). It will be noticed that the initial rate rapidly falls off so that, instead of the usual straight line, a curve is obtained. Drying has little or no effect, and in both cases the results are practically negligible when compared to the morphologically similar aerobic spore-bearers *B. subtilis* and *B. megatherium*. Duplicate results of *B. sporogenes* are seen in the controls of Figs. 23 and 24.

IV. *Aerobic Cocci.*

11. *Staphylococcus aureus* was grown in six Roux bottles of broth for two days. Fig. 17 shows the large uptake of oxygen of *Staph. aureus*, which is only slightly affected by drying.

12. *Sarcina aurantiaca* was grown on agar plates for three days. Fig. 18 shows the oxygen uptake, which is somewhat reduced on drying. Fig. 19 shows the prolonged steady oxygen uptake of this organism, no diminution in the rate appearing during a period of 10 hours.

13. *Streptococcus acidi lactici* was grown for two days in 18 Roux bottles of glucose broth. Fig. 20, which is drawn to the scale used for *B. sporogenes* (Fig. 16), shows that in *S. acidi lactici* the oxygen uptake is almost negligible. Duplicate results are to be found in the controls of Figs. 21 and 22.

It is most striking to compare this result with those of the other aerobic cocci, *Staphylococcus* and *Sarcina*, and to find that, instead of resembling these organisms, *S. acidi lactici* gives an oxygen uptake that is almost exactly equal to that of the anaerobic *B. sporogenes*. In addition to the fact that *S. acidi lactici* and *B. sporogenes* both take up only a minute amount of oxygen, these organisms also both show rapid reduction in the rate of oxygen uptake, unlike all the other bacteria investigated.

Effect of addition of catalase on oxygen uptake of bacteria which lack catalase.

Previous investigations [Callow, 1923] had revealed the fact that *S. acidi lactici*, like the anaerobes, contained no catalase, whereas all the other aerobes mentioned in this paper contained catalase. On this account it was thought that possibly the addition of catalase to *S. acidi lactici* or to *B. sporogenes* might lead to an increase in oxygen uptake, or at any rate to a reduction in the falling-off that is so characteristic of the rate of uptake of these two organisms.

Preparation of Sterile Catalase. Ox liver was minced finely, ground up with distilled water and filtered. Alcohol was added to the clear filtrate until no more precipitation occurred. The precipitate was filtered off, washed with absolute alcohol and dried *in vacuo*. The dry powder was dissolved in distilled water, filtered and passed through a bacteriological filter. The resulting clear liquid contained active catalase but gave no peroxidase reaction with benzidine and either H_2O_2 or ethyl hydroperoxide.

For control experiments the enzyme was inactivated by boiling for five minutes.

S. acidi lactici was washed as usual and emulsified in buffer but, instead of the usual 3 cc., 2·5 cc. were placed in each of three Barcroft bottles. To one was added 0·5 cc. sterile catalase solution, to another 0·5 cc. inactivated "catalase" solution, and to the third 0·5 cc. sterile distilled water to act as a control.

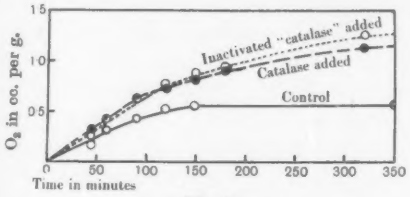

Fig. 21.
S. acidi lactici with addition of catalase.

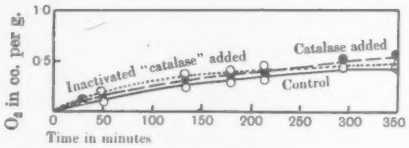

Fig. 22.
Dried *S. acidi lactici* with addition of catalase.

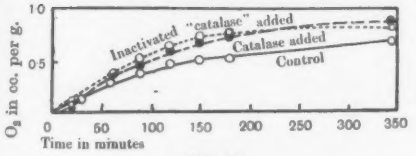

Fig. 23.
B. sporogenes with addition of catalase.

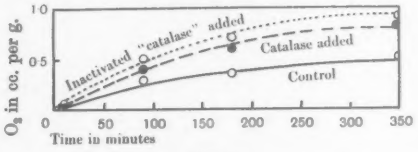

Fig. 24.
Dried *B. sporogenes* with addition of catalase.

Fig. 21 shows that, before the organism was dried, the liver extract supplied a certain amount of readily oxidisable material which caused a slight increase in the oxygen uptake, but clearly the catalase had no specific effect, as the inactivated enzyme gave exactly the same result. Fig. 22 shows that in the case of the dried *Streptococcus* there was no increase in oxygen uptake with the addition of either catalase or inactivated "catalase."

Figs. 23 and 24 show that *B. sporogenes*, like *S. acidi lactici*, is unable to distinguish between boiled and unboiled liver extract, thus showing that under these conditions the addition of catalase has no effect on the oxygen uptake.

Discussion of Results.

Setting aside for the moment the exceptional case of *S. acidi lactici*, it must be remarked that the oxygen uptake of aerobic bacteria is large when compared with that of mammalian tissue. The bacteria examined took up 5–25 cc. oxygen per hour per g. of dry weight. Meyerhof [1919] found that 1 g. of intact frog muscle at 22° took up 30–48 c.mm. oxygen in one hour. Assuming that the frog muscle contained 70–75 % water, the oxygen uptake per g. of dry weight would be at most 152 c.mm. as against 5–25 cc. for the same weight of bacteria in the same time. After cutting up the frog muscle very finely, Meyerhof found that the oxygen uptake was increased to 400–540 c.mm. which would give at most 2·16 cc. per hour per g. of dry weight, which is less than half the amount of oxygen taken up by the least efficient (considered from this point of view) bacterium. The investigation of the oxygen uptake of bacteria after drying and then emulsifying in buffer yielded several interesting points. The *Timothy Grass Bacillus*, an acid-fast organism which is characterised by its fatty envelope, actually gave the same oxygen uptake as before. Several other organisms, such as *B. alcaligenes* and *Staph. aureus*, were hardly affected by the process of drying while others, such as *B. pyocyaneus*, showed considerably reduced uptake of oxygen after having been dried. This point is of great interest in connexion with the work of Shattock and Dudgeon [1912]. These authors dried certain non-sporing bacteria in a charcoal liquid air vacuum, allowing controls to dry in the air for the same length of time. They found that, in the case of *B. coli* and *B. typhosus*, the bacteria were dead after 4–6 days in the vacuum tubes, and the air dried controls were dead on the fourth day, that is to say the presence of air had little effect on the viability. *Staph. aureus* was found to be more resistant to desiccation both in the vacuum tubes and in air. *B. pyocyaneus* however was exceptional as it survived for more than six months if kept in a vacuum tube, while the air control was never alive after the ninth day. The authors suggest that the bacillus may be killed "by a recurrent process of oxidation." It must be remembered that the process of drying used in the present investigation (in vacuum desiccator over H_2SO_4) does not exclude the presence of air, so that the dried *B. pyocyaneus* in this case would correspond to the air-dried control of Shattock and Dudgeon, which they found to be rapidly affected. This fits in exactly with the results of oxygen uptake, which were much lower on drying in the case of *B. pyocyaneus*, pointing to the fact that this organism has a high content of readily oxidisable material which is not protected against the action of atmospheric oxygen.

Unlike the majority of aerobic bacteria, which take up large amounts of oxygen at a rate which shows no diminution even after 10 hours, the anaerobe *B. sporogenes* only takes up a minute quantity of oxygen, and this at a rapidly decreasing rate. The same small and rapidly decreasing uptake is actually found in the aerobe *S. acidi lactici*, which in this respect behaves like an

anaerobe. This organism in fact appears to bridge the gulf between aerobes and anaerobes. Like an aerobe it can grow aerobically on the surface of an agar slope, an action that no anaerobe can perform, and yet like an anaerobe it has no catalase and is only capable of taking up small quantities of oxygen. The oxygen uptake of bacteria is presumably due to the action of enzymes on the oxidisable material present, and in the case of those bacteria which have only a small oxygen uptake there appear to be three possibilities; either there is lack of suitable enzymes, or lack of available oxidisable material, or some product of oxidation rapidly inhibits the reaction, as for example the accumulation of H_2O_2 in the non-catalase bacteria [see McLeod, 1922]. This last possibility has been investigated in the case of *B. sporogenes* and *S. acidi lactici*, both of which lack catalase and are only able to take up a very small amount of oxygen, at a rapidly decreasing rate. A sterile preparation of liver catalase was added to each of these organisms before and after drying, but in no case was there any increase in oxygen uptake other than that given by addition of the control inactivated "catalase," which provided a small amount of readily oxidisable material. Of course this negative result may merely mean that these bacterial cells are not permeable to liver catalase, and that this enzyme cannot be utilised unless it enters the cell. However, this and other observations mentioned in a former paper [Callow, 1923] point to the fact that possibly some factor other than catalase is of importance here, either in connexion with catalase or alone. It is of course possible that the lack of catalase in these organisms is not connected with the remarkably low oxygen uptake. Further work on this point is in progress.

In conclusion, the author gladly takes this opportunity of thanking Prof. F. Gowland Hopkins for his interest during the progress of this work.

Summary.

1. Bacteria were washed free from culture medium, emulsified in sterile buffer solution, p_H 7·6, and investigated for oxygen uptake at 22° in Barcroft's micro-respirometer.

2. Variations in p_H of the buffer solution, 6·6 to 8·6, had no appreciable effect on the oxygen uptake, nor had the replacement of the phosphate buffer solution by distilled water.

3. Bacteria grown on agar or glucose tryptic broth gave the same oxygen uptake as when grown on tryptic broth.

4. *B. pyocyaneus*, *B. fluorescens*, *B. prodigiosus*, *B. alcaligenes*, *B. proteus*, *B. coli*, *Timothy Grass Bacillus*, *B. megatherium*, *B. subtilis*, *Staph. aureus* and *Sarcina aurantiaca* took up large amounts of oxygen (5 to 25 cc. per hour per g. of dry weight) at a steady rate.

5. Bacteria were dried *in vacuo*, emulsified in buffer and examined for oxygen uptake. *B. alcaligenes* and *Staph. aureus* took up nearly as much

oxygen after drying as before, and the acid-fast *Timothy Grass Bacillus* gave the same result dried as normal. In *B. pyocyaneus* the oxygen uptake was very much reduced on drying.

6. The aerobe, *S. acidi lactici*, resembled the anaerobe, *B. sporogenes*, in taking up only a minute amount of oxygen at a rapidly decreasing rate (less than 0·5 cc. per g. of dry weight in the first hour, and practically none in the succeeding hours).

7. Addition of sterile catalase solution had no effect on the oxygen uptake of *S. acidi lactici* or *B. sporogenes*.

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LXX. THE EQUILIBRIA EXISTING BETWEEN SUCCINIC, FUMARIC, AND MALIC ACIDS IN THE PRESENCE OF RESTING BACTERIA.

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It was observed in a series of preliminary experiments described in a previous paper [Quastel, 1924] that the rate of decolorisation of methylene blue by resting *B. coli* and *B. pyocyaneus* was considerably accelerated by the presence of sodium succinate. These observations have been extended with the result that it is clear that a reversible equilibrium

succinic acid + methylene blue $\leftrightharpoons$ fumaric acid + *leuco*-methylene blue

exists in the presence of these resting organisms.

By resting organism we mean an organism which has been grown for two days in tryptic broth [Cole and Onslow, 1916], separated by centrifuging, thoroughly washed with normal saline, made up to a thick emulsion with saline, and finally very well aerated, which serves to produce a fairly homogeneous emulsion and to remove any traces of easily oxidisable material. Such an emulsion has been used for the investigations to be described and is always employed under such conditions that growth does not occur. The reactions brought about by the organism under these conditions we consider to be reactions produced by the resting organism. We have hesitated in committing ourselves to the term "resting organism" in view of the designation of the sporing stage of certain organisms as a resting stage. We feel, however, that no confusion need necessarily arise. The term resting organism as we have defined it is useful for comparison with resting muscle and other tissues.

Our investigations have been confined so far almost exclusively to resting *B. coli comm.*, but qualitative experiments show that resting *B. pyocyaneus* has similar properties.

Measurements of the velocity of reduction of methylene blue were made under anaerobic conditions in vacuum tubes such as those used by Thunberg [1920] and later by Hopkins and Dixon [1922]. The succinic, fumaric and malic acids were always neutralised with caustic soda to a p_H of 7·2. Care was taken that the solutions under investigation were properly buffered. The observations which are recorded below are typical of the average of those made. They are only a small proportion of the total number.

Our first inquiries turned on the properties of the enzyme, existing in the resting organism, which is capable of transferring hydrogen from succinic

acid to methylene blue. We found that the activity of the organism varied considerably with its history, generally becoming more marked after standing for one or two days. If allowed to stand too long, the organism itself acquires increased reducing properties which make it undesirable for use. We have employed two strains of *B. coli* and several growths from each strain. These will be referred to as coli 1, coli 2, etc. In all cases, the reduction velocities can only be regarded from a comparative point of view, since the various emulsions differed in the total quantity of organism present, but for any one series of experiments the same emulsion was always used.

As instances of the reducing activity of the organism with and without succinic acid, the following observations are typical.

Tubes...	1	2	3	4
cc. of organism (coli 1) ...	1	1	1	1
„ 7·6 buffer	1	1	1	1
„ 1 % sodium succinate...	1	0	1	0
„ water	0	1	0	1
„ methylene blue (1/5000)	1	1	1	1
Temperature	38°	38°	60°	60°
Time for decolorisation ...	35 mins.	Not decolorised in 16 hrs.	6 mins.	1 hour

Potassium cyanide and sodium fluoride do not inhibit the reduction of methylene blue by the organism in presence of succinate.

Effect of hydrogen ion concentration.

It was at once evident from observations on the effect of varying hydrogen ion concentration on the times of reduction that two effects were superimposed upon one another, (1) an effect upon the physical structure of the organism, (2) an effect on the chemical reaction. Below a p_H of 5·0 no apparent reduction could be obtained, and the organism appeared to be coagulated. Above p_H 6·0 there was rapid increase of the velocity of reduction with decrease of hydrogen ion concentration until a p_H of 9·0, when a further increase in p_H did not bring about any rise in reaction velocity. We have not investigated the effect of a p_H above 10·0, but we have reason to believe that in alkaline solutions the organism suffers some change, which affects its reducing activity considerably. The most suitable p_H for a high reduction velocity without fear of inactivation of the organism is about 7·4.

The results of a series of experiments on the effect of hydrogen ion concentration on reduction times are expressed graphically in Fig. 1. The solutions used consisted of 1 cc. of organism (coli 2), 1 cc. of 1 % sodium succinate, 1 cc. of methylene blue (1/5000), and 1 cc. of buffer of varying p_H. The temperature throughout was 45°.

It is interesting to compare these results with those of Ohlsson [1921] who finds with succinodehydrogenase extracted from horse muscle an optimum p_H of about 9·0 above which there is a sharp decrease in activity. The enzyme used by Ohlsson was thus more sensitive to low hydrogen ion concentrations than the resting organism.

When phosphate buffer was replaced by borate buffer of the same p_H, there was no difference in the velocity of reduction.

That each emulsion of organism was homogeneous and that equal volumes could be regarded as possessing the same quantity of enzyme was demonstrated by the fact that reduction times of equal volumes of emulsion for the same quantities of methylene blue and succinate were identical.

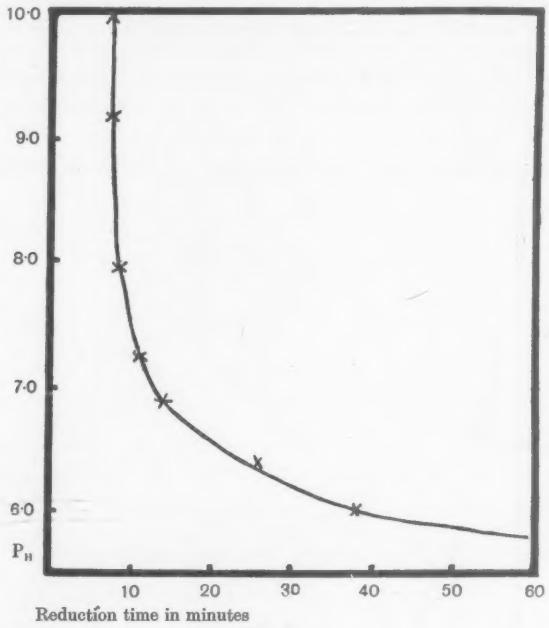

Fig. 1.

Effect of temperature at constant hydrogen ion concentration.

Here, as in the previous experiments, two superimposed effects (1) on the physical condition of the enzyme, (2) on the chemical reaction, are obvious. The reduction velocity increases with increase of temperature, the temperature coefficient between 30° and 60° being 2·1 (see Fig. 2). At 70° there is an extremely rapid reduction for two or three minutes, which suddenly ceases before complete decolorisation, and this must be associated with the fact that the organism has become coagulated. It appears, therefore, that at this temperature, the increased velocity of destruction of the enzyme (that is, of retardation of reduction) becomes of the same order as the increased velocity of the reaction between the succinate and the methylene blue. The following observations on the effect of exposing the resting organism to a high temperature will make this clear.

Tubes...	1	2	3	4
cc. of organism (coli 3)	1	1	1	1
„ 7·6 buffer	1	1	1	1
„ 1 % sodium succinate	1	1	1	1
„ methylene blue (1/5000)	1	1	1	1
Temperature at which organism was previously heated for 10 mins. ...	60°	—	70°	—
Temperature of thermostat	60°	60°	60°	60°
Time for decolorisation with heated organism	9 mins.	—	No decolorisation	—
Time for decolorisation with unheated organism (control)	—	4¾ mins.	—	4 mins. 40 secs.

We find that a temperature of 45° is very suitable for a high velocity of reduction without risk of injury to the organism, and all subsequent work was consequently carried out at this temperature.

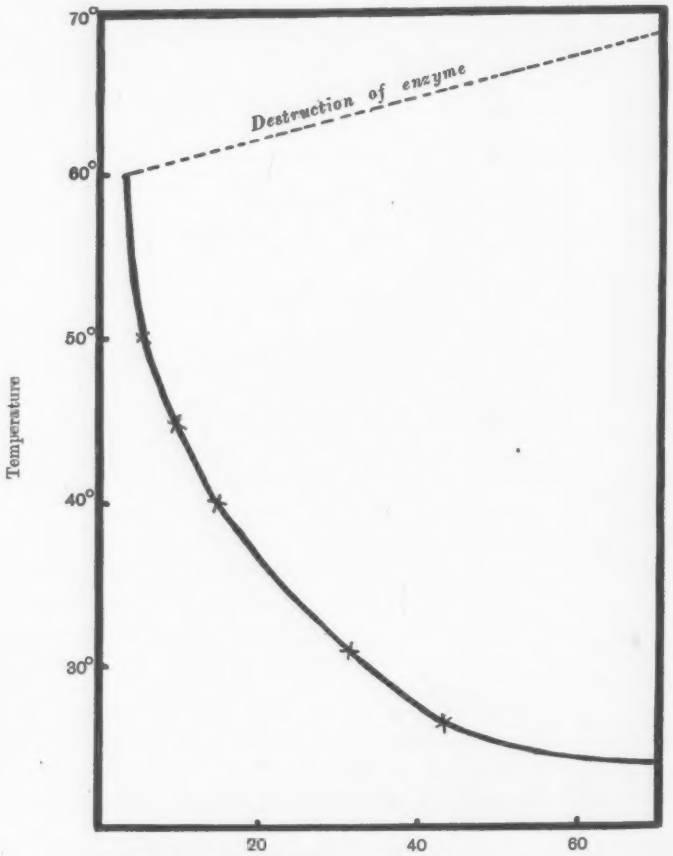

Fig. 2.

Effect of concentration of organism.

The rate of reduction is roughly directly proportional to the concentration of organism present.

Tubes...	1	2	3	4
cc. of organism (coli 4) ...	3	2	1	0·5
„ 7·3 buffer	1	1	1	1
„ 1 % sodium succinate...	1	1	1	1
„ methylene blue (1/5000)	1	1	1	1
„ saline	0	1	2	2·5
Time for decolorisation ...	2½ mins.	3½ mins.	7 mins.	13 mins.

All attempts to extract a succinodehydrogenase from the organism by the means which have been successful in the case of muscle have so far failed. Shaking the organism with $N/200$ NaOH or with $M/15$ $(NH_4)_2HPO_4$ is not effective in removing the reducing factor.

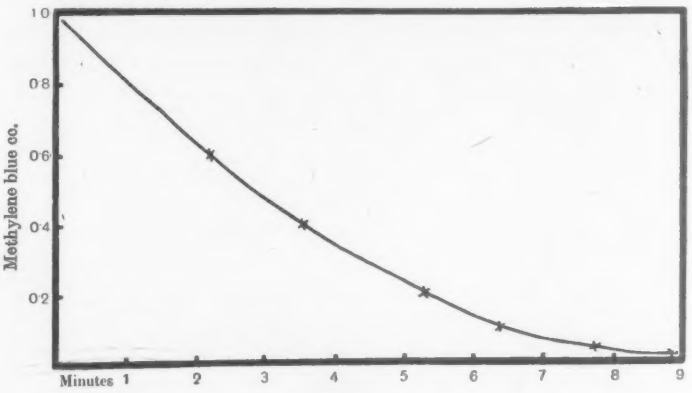

Fig. 3.

The rate of decolorisation of methylene blue.

Fig. 3 is a typical example of the results obtained in a series of experiments on the rate of reduction of methylene blue by sodium succinate. A mixture of 1 cc. of organism, 1 cc. of 7·2 buffer, 1 cc. of 1 % sodium succinate, and 1 cc. of methylene blue (1/5000) was placed in a vacuum tube and allowed to reduce under anaerobic conditions at 45°. The methylene blue was estimated at intervals by colorimetric comparison with standard solutions of methylene blue, to which organism had been added in order to make the opacities of the vacuum tube and the comparison tube as nearly equal as possible.

The rate of decolorisation of a solution *in vacuo* was not different from that of one in an atmosphere of nitrogen.

Reversibility.

It was shown in an earlier communication [Quastel, 1924] that fumaric acid (as the sodium salt) provides a very efficient nutrient supply to *B. pyocyaneus* and *B. coli* and it was shown too that, in the case of the former

organism, pyruvic acid was the first demonstrable product in the fermentation, oxalacetic acid being the most likely precursor of pyruvic acid. Evidence was brought forward to demonstrate that the unsaturated linkage was capable of fixing oxygen to produce a ketonic structure, the process being extremely rapid with the growing organism. Now it seemed possible that this process might be made to occur anaerobically by using a suitable hydrogen acceptor instead of atmospheric oxygen; that fumaric acid and, say, methylene blue, might act as oxygen acceptor and hydrogen acceptor respectively with water as the donator of both the oxygen and the hydrogen. This was tested, and it was found that fumaric acid in presence of the resting organism produced no reduction of methylene blue whatever. What was actually found was a definite inhibition of the normal reducing capacity of the organism alone. Investigating this inhibition further, it was found that fumaric acid inhibits the reduction of methylene blue by sodium succinate in presence of resting organism, in much the same way as has been demonstrated by Wishart [1923] for the case of muscle. No claims have been made by the workers on resting muscle that an equilibrium exists between succinic acid, fumaric acid, methylene blue, and its leuco-derivative, but our observations on the relative degrees of inhibition exerted by varying concentrations of fumarate led us to the belief that a true, reversible equilibrium does exist. This opinion was strengthened, moreover, by finding that maleic acid has no similar effect to fumaric acid, which at once gives the fumaric inhibition a specific character. Concentrations of maleic acid (neutralised by sodium hydroxide) varying from 0·1 % to 10 % had no apparent effect on the decolorisation of methylene blue by succinate in presence of resting organism.

The fumaric acid inhibition was then investigated in the following ways: determination of the effect of sodium fumarate on *leuco*-methylene blue, (1) with resting organism, (2) with dead organism, (3) without organism.

(1) In order to show that the inhibition was actually due to a reaction between fumarate and leuco-methylene blue, the following experiment was devised. Use was made of the fact that the resting organism is capable of reducing oxidised glutathione to the –SH compound, the reduced glutathione itself reducing methylene blue. Thus in presence of a trace of oxidised glutathione the resting organism very readily reduces methylene blue. If now to the system—resting organism, oxidised glutathione, and methylene blue—sodium fumarate be added (in excess of the glutathione present), the methylene blue is no longer completely reduced. The following experiment demonstrates this.

Tubes...	1	2	3
cc. of organism (coli 5)	2	2	2
„ 7·2 buffer	1	1	1
„ methylene blue (1/5000) ...	0·5	0·5	0·5
„ 1 % neutral oxidised glutathione	—	0·5	0·5
„ 1 % sodium fumarate	—	—	1·0
„ of saline	1·5	1·0	—
Time for decolorisation	Not decolorised	11 mins.	Not completely decolorised in 1 hr.

Similarly, sodium fumarate has an inhibitive action on the reduction of methylene blue by cysteine in presence of resting organism.

Tubes...	1	2
cc. of organism (coli 5)	1	1
„ 7·2 buffer	1	1
„ methylene blue (1/5000) ...	1	1
„ neutral cysteine solution (0·3 %)	0·5	0·5
„ 1 % sodium fumarate	—	1
„ saline	1	—
Time for decolorisation	15 mins.	Not completely decolorised in 1 hr.

The most conclusive manner of demonstrating the oxidising action of sodium fumarate on *leuco*-methylene blue in presence of resting organism is to allow a solution of sodium fumarate to react *in vacuo* with a mixture containing the *leuco*-dyestuff and the resting organism. The white solution gradually becomes bluer until it reaches a definite intensity of colour depending on the concentration of fumaric acid, and this colour remains practically permanent. The experiment is best done by making use of an inverted U tube which can be evacuated. The two limbs of the U tube employed were 7″ by $\frac{3}{4}$″, the neck being 3″ long. The neck was plugged by a short piece of rubber tubing containing a length of glass tube to which was affixed an air-tight tap. The tap was always well greased and a vacuum could be maintained almost indefinitely in the tube. In one limb of the tube a mixture of resting organism, buffer solution, sodium succinate solution, and methylene blue is placed, in the other limb a solution of sodium fumarate. If care be taken in the quantities of material used, in the evacuation, and in the subsequent proceedings, there need be no bubbling of the solution in one limb into that in the other. The risk of the solutions mixing by ebullition may be, however, completely eliminated by filling the evacuated tube with nitrogen. The evacuated (or nitrogen filled) tube is left in the thermostat until reduction of the methylene blue by the succinate has been accomplished. The two solutions are then mixed and in a minute or two the gradual blueing of the solution becomes apparent. Sodium maleate has no such effect. If the evacuation has not been sufficiently complete, there may be a slight immediate blueing, due to a trace of oxygen left in the solution. On standing for about five minutes, however, this colour disappears as the succinate exerts its reducing power. A solution which has become blue owing to addition of sodium fumarate remains blue for an almost indefinite period.

(2) It was then decided to ascertain whether the reaction between the *leuco*-methylene blue and the fumarate depended on a thermolabile enzyme. Use was again made of the vacuum U tube. In one limb the succinate + resting organism + methylene blue mixture was placed as before, and in the other the solution of fumarate. The tube was evacuated, filled with nitrogen, and allowed to stand in the thermostat until reduction of the methylene blue was accomplished. It was then placed in a water-bath and the temperature of the bath was gradually brought up to 100°. The pressure

of gas was relieved by opening the tube to atmospheric pressure at intervals. After exposure to this temperature for a quarter of an hour, the tube was allowed to cool, the tap being closed so that no oxygen entered as cooling proceeded. The tube was then re-evacuated to remove any traces of oxygen that might have entered, the solutions in the two limbs were mixed, and the tube was placed in the thermostat. No blueing whatever occurred.

(3) Definitely to show that there was no action between sodium fumarate and *leuco*-methylene blue, the following experiment was performed. In one limb of the vacuum U tube was placed a mixture of cysteine solution, buffer solution, and methylene blue, and in the other sodium fumarate solution in a concentration considerably greater than that of the cysteine. The tube was evacuated and placed in the thermostat, and when the methylene blue had been reduced by the cysteine, the solutions were mixed. There was no oxidation of the *leuco*-methylene blue. When this experiment was repeated in the presence of resting organism, a slight but definite blueing of the *leuco*-methylene blue did occur.

It must be concluded from these experiments that just as sodium succinate reduces methylene blue in the presence of resting organism, so does sodium fumarate oxidise *leuco*-methylene blue in presence of the organism.

The matter was investigated from a quantitative aspect. It was desirable to show first that the equilibrium attained by a mixture of known quantities of succinate, fumarate, and methylene blue, was the same as that reached after the addition of the same quantity of fumarate to a solution of the same quantities of methylene blue and succinate, complete reduction of the dyestuff having been allowed to occur. In a vacuum tube was placed the following mixture: 1 cc. coli 6, 1 cc. 7·2 buffer, 1 cc. methylene blue (1/5000), 0·25 cc. 1 % sodium succinate, 1 cc. 1 % sodium fumarate, and the rate of decolorisation was determined by colorimetric comparison. Again, in one limb of a vacuum U tube was placed the mixture of 1 cc. coli 6, 1 cc. 7·2 buffer, 1 cc. methylene blue (1/5000), 0·25 cc. 1 % sodium succinate, and in the other limb 1 cc. 1 % sodium fumarate. Reduction of the methylene blue was allowed to proceed to completion, the solutions in the two limbs were mixed, and the rate of blueing observed by colorimetric comparison. The results are expressed graphically in Fig. 4. It will be seen that the same equilibrium point is approached asymptotically by both solutions.

Assuming that the equilibrium

succinic acid + methylene blue $\rightleftharpoons$ fumaric acid + *leuco*-methylene blue

exists in presence of the resting organism, it follows that at equilibrium conditions will be represented by

$$\frac{[\text{fumaric acid}]\,[\text{leuco-methylene blue}]}{[\text{succinic acid}]\,[\text{methylene blue}]} = K \text{ (equilibrium constant)},$$

where the square brackets denote molecular concentrations at equilibrium. If we begin with a molecules of sodium succinate, b of methylene blue, and

x of sodium fumarate, and if at equilibrium there are n molecules of methylene blue present, we have

$$\frac{an - n(b-n)}{x(b-n)+(b-n)^2} = \frac{1}{K}.$$

This equilibrium constant will be independent of the amount of resting organism so long as conditions of true catalysis hold. If, however, some material of the resting organism enters into one side of the equation and not into the other, or if different quantities of the same material enter into the two sides of the equation, K will vary with the quantity of organism present.

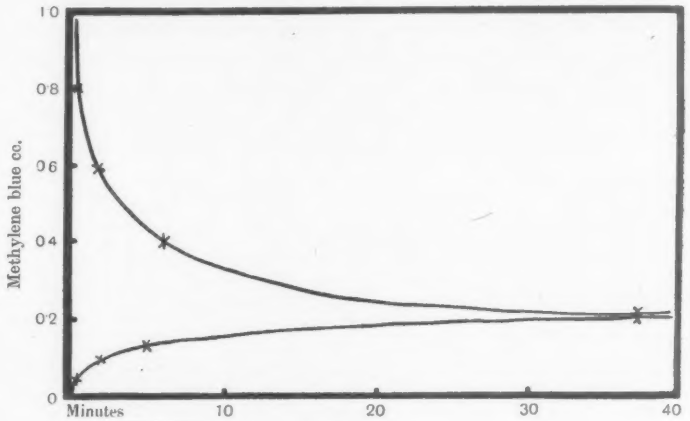

Fig. 4.

A large number of observations were made on the amounts of methylene blue left at equilibrium with varying initial quantities of succinate, fumarate, organism, and methylene blue. The hydrogen ion concentration and temperature were kept constant throughout. The solutions were mixed in a vacuum tube, evacuated as quickly as possible, and left in the thermostat for one hour, when it was found that equilibrium had been reached. Generally, with an active preparation of *B. coli*, ten to twenty minutes were really sufficient. The rates at which equilibrium was reached varied with the quantities of organism present. The methylene blue was estimated by colorimetric comparison, the method being to add a known quantity of methylene blue to a definite quantity of the resting organism in a tube precisely similar to the vacuum tube. Water was then added to the comparison tube from a burette until the intensity of colour of methylene blue in the vacuum and comparison tubes was the same. Knowing the total volumes in both tubes, it is easy to calculate the amount of methylene blue in the vacuum tube. Control experiments showed that with practice at matching the intensities of colour the maximum error does not exceed 5 %. The greatest errors occur with very high and very low concentrations of methylene blue. The difficulty

in matching the colours lies chiefly with the opacity due to the organism and care must be taken to make the opacities roughly the same by addition of suitable known quantities of organism to the comparison tube. It is difficult to obtain accurate readings with *B. pyocyaneus* owing to the pigment in this organism. *B. coli* if it has been properly washed has only the very slightest yellowish tinge which does not affect readings appreciably. It may be mentioned here that *B. coli* is far easier to handle than *B. pyocyaneus* from the point of view of physical characteristics and this constituted one of the reasons for our dealing almost exclusively with *B. coli*.

Table I gives examples of the results obtained with varying concentrations of succinate, fumarate, methylene blue, and organism. The percentage reduction found and that theoretically deduced for a definite value of K is given in each case. We have taken $K = 3{\cdot}0$ and it will be seen that the agreement between the observed and the calculated reductions is sufficiently good to prove that the equilibrium postulated above does actually exist.

Table I. *Temperature throughout* = 45°. $p_H = 7{\cdot}2$, $K = 3{\cdot}0$.

Amount of organism	cc. of 1 % sodium succinate	cc. of 1 % sodium fumarate	cc. of methylene blue (1/5000)	cc. of methylene blue remaining at equilibrium	% reduction found	% reduction calculated
2 cc. coli 6	0·1	1·0	0·75	0·58	22·6	22·0
,,	0·25	1·0	0·8	0·467	41·7	42·0
,,	0·25	1·0	1·0	0·583	41·7	42·0
,,	0·5	1·0	0·5	0·207	58·6	59·8
2 cc. coli 7	1·0	1·0	1·0	0·245	75·5	75·0
,,	1·0	0·5	1·0	0·166	83·6	85·7
,,	1·0	0·75	1·0	0·202	79·8	80·0
,,	1·0	1·5	1·0	0·515	68·5	66·6
1 cc. coli 7	1·0	1·0	1·0	0·260	74·0	75·0
,,	0·75	1·25	1·0	0·380	62·0	64·4
,,	0·5	1·5	1·0	0·50	50·0	50·0
,,	1·5	0·5	1·0	0·08	92·0	90·0
,,	2·0	1·0	1·0	0·124	87·6	85·7
2 cc. coli 7	0·5	1·5	1·0	0·50	50·0	50·0
,,	1·5	0·5	1·0	0·09	91·0	90·0
,,	0·25	1·75	1·0	0·67	33·0	30·0
,,	1·25	0·75	1·0	0·17	83·0	83·3
1 cc. coli 8	1·0	3·0	1·0	0·486	51·4	50·0
,,	1·0	2·0	1·0	0·412	58·8	60·0
,,	1·0	0·25	1·0	0·104	89·6	92·3
2 cc. coli 8	*5·0	1·0	2·5	0·066	97·2	93·6
,,	*5·0	1·5	2·5	0·176	92·9	92·3
,,	*5·0	0·0	15·0	0·183	98·7	99·0
,,	*2·5	2·0	2·5	0·494	80·2	79·2
,,	*2·5	2·0	5·0	0·444	91·1	89·6

* In these cases more concentrated solutions of sodium succinate and methylene blue were used.

It will be seen on examination of the table that K is independent of the quantity of organism present.

Isolation of fumaric acid.

For isolation of fumaric acid from succinic acid and methylene blue in presence of organism the following method proved to be satisfactory. The

resting organism (*B. coli*) from a 48 hours' growth on three Roux bottles of tryptic broth (each containing 150 cc.) was placed in a vacuum flask with 50 cc. of a phosphate buffer at p_H 7·2, and solutions containing 2 g. of sodium succinate, and 1 g. of methylene blue, the total volume being 350 cc. The flask was evacuated and left at 37° for 24 hours. After this time the flask was opened and Merck's charcoal was added. The solution was filtered, the residue well washed and the filtrate and washings evaporated to low bulk on the water-bath. The liquid was acidified with sulphuric acid (to 10 % acid) and extracted well with ether. The ethereal extract was filtered through dry paper and evaporated. The crude acids which were left as residue were dissolved in a small quantity of water and to the solution was added silver nitrate solution. The white precipitate was well washed, suspended in warm water, and treated with sulphuretted hydrogen. The silver sulphide was filtered off, and the filtrate evaporated to dryness. About 80 mg. of crude acid were obtained from which 60 mg. of a pure specimen of fumaric acid were prepared. This does not represent the total yield of fumaric acid, for the method given above, though fairly satisfactory, is not strictly quantitative. The fumaric acid was identified by its melting point and a mixed melting point with a pure specimen of fumaric acid. It did not melt at 200° in an open melting point tube but showed signs of sublimation. In a closed melting point tube it melted at 280°. Its behaviour was not altered by admixture with an authentic specimen of fumaric acid. A known quantity was titrated with standard alkali from a micro-burette: 0·0143 g. of the acid dissolved in water required 2·00 cc. of 0·1163 *N* alkali for neutralisation, a quantity equivalent to 0·0145 g. of fumaric acid. The acid was precipitated from aqueous solution by silver nitrate solution and decolorised potassium permanganate in the cold.

It was further identified by making use of the equilibrium equation given above. 0·0052 g. of the acid was neutralised with caustic soda and added to a mixture of 2 cc. coli 9, 1 cc. 7·2 buffer, 1 cc. methylene blue (1/5000), 0·2 cc. 1 % sodium succinate, in a vacuum tube. The tube was evacuated and the equilibrium point reached was estimated by colorimetric comparison of the methylene blue. This was equivalent to 0·23 cc. of 1/5000 methylene blue, which, on substituting in the equation above with $K = 3{\cdot}0$ gives 0·0049 g. of fumaric acid.

Malic acid.

On examining the effect of malic acid (as the sodium salt of *l*-malic acid) on the reduction of methylene blue in presence of the resting organism it was found

(1) that malic acid resembles fumaric acid in not reducing methylene blue in presence of resting organism;

(2) that malic acid slightly but definitely inhibits the decolorisation of methylene blue by succinate in the presence of resting organism.

The following experiments demonstrate this.

(1) Tubes...	1	2	3	4	5
cc. of organism (coli 10) ...	1	1	1	1	1
„ 7·2 buffer	1	1	1	1	1
„ methylene blue (1/5000)	1	1	1	1	1
„ 1 % sodium succinate...	—	0·5	—	0·5	0·5
„ 1 % malic acid (as sodium *l*-malate) ...	—	—	1	0·5	—
„ 1 % sodium fumarate ...	—	—	—	—	0·5
„ saline	1	0·5	—	—	—
Time for decolorisation ...	Not decolorised	12 mins.	Not decolorised	Nearly decolorised in 12 mins., colour apparently stable	Reaches equilibrium

(2) Tubes...	1	2
cc. of organism (coli 10)	1	1
„ 7·2 buffer	1	1
„ methylene blue (1/5000)	1	1
„ 1 % sodium succinate	0·2	0·4
„ 1 % malic acid (as sodium *l*-malate)	1	1
Equilibrium reached (expressed in cc. of 1/5000 methylene blue)	0·17	0·07 (observed after 33 mins.)

It will be shown in a subsequent communication that *B. pyocyaneus* ferments malic in the same manner as fumaric acid—pyruvic acid being produced. Emmerling and Reiser [1902] have found fumaric acid among the products of fermentation of malic acid by *B. fluorescens*. In the case of muscle, it has been proved that fumaric acid is convertible to malic acid [Einbeck, 1919], the laevo-modification being formed [Dakin, 1922]. It would seem therefore that an equilibrium between fumaric acid and *l*-malic acid would be likely to occur with resting bacteria. Investigation showed that a solution of sodium fumarate in presence of resting *B. coli* gave rise to a solution with a laevo-rotation, and that sodium *l*-malate gave rise to fumaric acid. The crop of *B. coli* from three Roux bottles was added to 100 cc. of 1 % sodium fumarate and 20 cc. of p_H 7·2 buffer in a vacuum flask, which was evacuated and kept at 37° for 24 hours. The solution after filtration and concentration gave a laevo-rotation in presence of uranium acetate. A similar quantity of resting *B. coli* was incubated for 34 hours with 100 cc. of 1 % sodium *l*-malate and 20 cc. of p_H 7·2 buffer in an evacuated flask. The solution after this time showed a diminished laevo-rotation. It was filtered through kieselguhr and 50 mg. of fumaric acid were obtained by precipitation with silver from an aqueous solution of the crude acids obtained by acid ether extraction. The fumaric acid was identified in the same way as that obtained from succinic acid. In an open melting point tube it did not melt at 200° but showed signs of sublimation. Its melting point was 270° in a sealed melting point tube. 0·0050 g. dissolved in water required 0·68 cc. of 0·1136 *N* alkali for neutralisation (corresponding to 0·0049 g. fumaric acid). The aqueous solution was not optically active.

We have found only minute quantities of fumaric acid produced from malic acid by resting *B. coli*, the amounts depending on the activity of the

organism. That the laevo-variety of malic acid is preferentially attacked is shown by the fact that inactive malic acid yields a solution giving (with uranium acetate) a dextro rotation. It is clear that the rates of production of fumaric acid from malic acid and *vice versa* are extremely slow, even in presence of relatively large quantities of the organism. They are not comparable in order of magnitude with the velocities of these changes as produced by muscle tissue [Dakin, 1922]. On the other hand, the actual amount of the requisite enzyme in the resting organism in our experiments must be small compared with the amount present in the muscle used in Einbeck's or Dakin's experiments.

The slight inhibition by malic acid of the complete decolorisation of methylene blue by succinate in the presence of resting organism may possibly be ascribed to the production of a small quantity of fumarate.

Oxygen uptake.

It was of interest to ascertain whether oxygen was taken up by resting organism in presence of succinate and fumarate. The Barcroft differential apparatus was used and Tables II and III summarise the results. There is

Table II.

Absorption vessels	1	2	3
	1 cc. coli (10) 1 cc. buffer 7·2 1 cc. saline	1 cc. coli (10) 1 cc. buffer 7·2 1 cc. 1 % sodium succinate	1 cc. coli (10) 1 cc. buffer 7·2 1 cc. 1 % sodium fumarate
Time mins.	Oxygen absorbed (in cmm.)		
92	80·4	253·3	135·6
188	150·2	513·4	402·9
268	194·9	790·7	680·4

Temperature of bath = 35° C.

Table III.

Absorption vessels	1	2	3
	1 cc. *B. pyocyaneus* 1 cc. buffer 7·2 1 cc. saline	1 cc. *B. pyocyaneus* 1 cc. buffer 7·2 1 cc. 1 % sodium succinate	1 cc. *B. pyocyaneus* 1 cc. buffer 7·2 1 cc. 1 % sodium fumarate
Time mins.	Oxygen absorbed (in cmm.)		
70	369·7	458·2	515·9
103	571·6	823·4	948·0
164	855·7	1632·5	1926·2
209	1026·9	2319·8	2844·1

Temperature of bath = 35° C.

no doubt that both with *B. coli* and *B. pyocyaneus*, the absorption of oxygen by the resting organism is largely increased in the presence of either succinate or fumarate. This was to be expected from the results given in the previous paper [Quastel, 1924]. In spite of the fact that oxygen was taken up by sodium fumarate in presence of *B. pyocyaneus*, tests for the presence of pyruvic acid (the guaiacol and nitroprusside tests) proved completely negative. Now since pyruvic acid is undoubtedly formed by *B. pyocyaneus* growing in a fumarate medium, and since it is unlikely that the resting organism will have

a different mode of oxidising fumaric acid, it would seem that with the resting organism, pyruvic acid is more quickly dealt with than with the growing organism. This has yet to be proved, but it is reasonable to ascribe the failure to obtain evidence of the production of a ketonic acid by the resting organism to differences between the rates of oxidation of fumaric and pyruvic acids by the resting and growing organisms respectively. The non-appearance of a ketonic acid when *B. coli* is grown on a fumarate medium might thus be explained as due to differences between the rates of oxidation by growing *B. coli* and growing *B. pyocyaneus* and not to the absence in the former of a specific enzyme capable of producing the ketonic acid.

Discussion.

Four main points emerge from these investigations. The first point is the association of the enzyme with the structure of the resting organism. It is apparent from the effects of hydrogen ion concentration and of temperature on the rates of decolorisation of methylene blue by succinate that a close connection exists between the chemical activity and the structure of the organism.

There is in the second place the close analogy between fresh muscle and resting bacteria (as represented by *B. coli* and *B. pyocyaneus*). The existence in these bacteria and in muscle of an enzyme capable of oxidising the saturated molecule of succinic acid to the unsaturated one of fumaric acid makes it probable that the enzyme is one of widespread occurrence. The reaction too is one of extreme importance, for it demonstrates the ability of the cell to produce a most reactive structure—the unsaturated linkage—from a saturated molecule. The next step apparently is the linking of oxygen on to the unsaturated bond to produce a ketonic structure—from fumaric acid, oxalacetic acid. It might be conceived that the mechanism of this oxidation of fumaric acid lies through the intermediate formation of malic acid, by the addition of the elements of water, and the subsequent giving up of two atoms of hydrogen. Thus:

$$COOH.CH{:}CH.COOH \longrightarrow COOH.CH(OH).CH_2.COOH \longrightarrow COOH.CO.CH_2.COOH.$$

This, however, is extremely unlikely, for malic acid is incapable of donating hydrogen to methylene blue in presence of resting organism. If would also be expected on the above hypothesis that fumaric acid would slowly reduce methylene blue in the presence of resting organism: this does not occur. Again fumaric acid has been found among the products of fermentation of malic acid [Emmerling and Reiser, 1902], but no malic acid has been found among the products of fermentation of fumaric acid [Quastel, 1924].

Such a connection between the related unsaturated and hydroxy acids, *e.g.* fumaric and malic acids, is of significance when we consider it in the light of the general association in nature of these types of acids. The possibility of an equilibrium, for instance, between crotonic and β-hydroxybutyric acids, is suggested, and experiments are being made to test this point.

We have found that a number of substances besides succinic acid are capable of transferring hydrogen to methylene blue in presence of resting organism, and our next communication, we hope, will deal with these.

The third point which emerges is the reversibility of the reaction between succinic acid, methylene blue, fumaric acid, and *leuco*-methylene blue in presence of the resting organism. We have found no evidence with the resting organism for Thunberg's theory [1920] that fumaric acid is a donator of hydrogen and exists in equilibrium with acetylenedicarboxylic acid. This may possibly occur in the case of muscle—so far as we know the matter has not yet been thoroughly investigated—but in the case of resting bacteria all the evidence points to fumaric acid being an acceptor of oxygen in the sense that it links oxygen to its molecule and is not a hydrogen donator.

This brings us to the fourth point, which is the distinction which must be drawn between methylene blue and oxygen as hydrogen acceptors. Fumaric acid can "accept" oxygen and yet not transfer any hydrogen to methylene blue. This makes it clear that, either there are two distinct modes of biological oxidation (apart from the rôle of glutathione), or else that the hypothesis [Wieland, 1913] by which an oxidisable substance must itself be capable of giving up hydrogen to a suitable acceptor must be modified.

It is a point worthy of further mention that though the change succinic acid $\longrightarrow$ fumaric acid, is a good example of Wieland's view, this view would necessarily involve for the further oxidation of fumaric acid the addition of the elements of water to form malic acid and the donation by this acid of two atoms of hydrogen. We find no evidence under the conditions of our experiments for this procedure.

Summary.

1. Resting bacteria (*B. coli* and *B. pyocyaneus*) possess an enzyme, similar to that existing in muscle, which is capable of bringing about the reduction of methylene blue in presence of sodium succinate.

2. The reversible equilibrium

succinic acid + methylene blue $\leftrightharpoons$ fumaric acid + *leuco*-methylene blue

is set up in presence of the resting organism. The equilibrium constant of this equation has been obtained. Fumaric acid has been isolated from a succinic acid—methylene blue mixture.

3. The reversible equilibrium

fumaric acid $\leftrightharpoons$ *l*-malic acid

appears to exist in presence of resting organism. Fumaric acid has been isolated in small quantity from *l*-malic acid after the action of resting organism.

4. The effects of hydrogen ion concentration and of temperature on the reduction times of methylene blue by succinate in the presence of organism have been investigated and attention is drawn to the close association between chemical activity and physical structure of the organism.

5. It has been shown that fumaric acid readily absorbs oxygen in presence of resting organism, though it will not donate hydrogen to methylene blue. This is discussed in relation to Wieland's hypothesis.

We wish to offer our sincerest thanks to Prof. F. G. Hopkins, F.R.S., for his continual interest and encouragement in this work. The thanks of one of us (M.D.W.) are due to the Medical Research Council for a part time grant.

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LXXI. EXPERIMENTS ON RESPIRATION AND FERMENTATION.

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The researches of Batelli and Stern [1911] and of Meyerhof [1918 *et seq.*] on tissue respiration, and those of the latter on its relation to alcoholic fermentation, have led to the conception that the respiration both of surviving muscle and of "acetoned" yeast is controlled by a factor which also plays an essential part in the process of alcoholic fermentation. Harden and Young [1906] had shown that, in addition to potassium and phosphate ions, a soluble "co-enzyme" must be present to ensure the fermentation of glucose by yeast. Meyerhof stated that this co-enzyme was also essential as a "co-enzyme to respiration" and he named it the "respiration substance" [Meyerhof, 1919, 2]. In the case of the respiration of washed muscle or of washed "acetoned" yeast suspended in a boiled extract of tissue or yeast, it provided a general catalytic stimulus to the cell respiration as a whole, accelerating the oxidation of various oxidisable substances also present in the cell extract, by virtue of its property of acting as an oxygen carrier. It exerted a similar function during the course of alcoholic fermentation [Meyerhof, 1919, 2]. On the other hand he maintained [Meyerhof, 1920, 2] that when a muscle is chopped, but not washed, and suspended in a buffer solution or in Ringer, the oxygen consumption observed is due to the oxidation of the lactic acid formed during the chopping, to the practical exclusion of any other respiratory process.

In a previous paper [Holden, 1923] it was suggested that, in the case of washed frog muscle respiring in a yeast "boiled extract," the oxygen uptake should be attributed rather to the presence of oxidisable substances in the extract than to the action of any "co-enzyme to respiration" having a solely catalytic function. The necessity for assuming that a respiratory co-enzyme exists does not seem indeed to have been established.

The present paper describes the results of experiments in extension of those referred to above. The principle of the experiments is for the most part similar to that of those already described. "Boiled extract" of muscle or yeast was aerated in contact with washed muscle or with "acetoned yeast." The effects of the extract after aeration, on the oxygen uptake of washed muscle, or the fermenting power of washed "acetoned" yeast, were compared

with those of the original unaerated extract, samples of which were stored *in vacuo* at 0°.

The yeast extracts were more concentrated than those previously employed, being made by stirring baker's yeast in its own weight of boiling water and filtering off the insoluble residue. The muscle extracts were made by mincing the muscle rapidly, covering it with distilled water, heating rapidly to boiling with constant stirring, and filtering by suction. The extract was immediately adjusted to p_H 7·6, a portion being passed through a Berkefeld filter and stored *in vacuo* at 0° as before. When the aeration was performed at p_H 5·8 in contact with yeast, the stored sample was stored at that p_H.

The muscle used in the respiration experiments was finely chopped with scissors and washed as often as stated in the tables. Each washing was performed by shaking vigorously in a stoppered bottle with the volume of distilled water shown. The washing was continued for 20 minutes and the muscle residue was then filtered off on a small suction filter.

The "acetoned" yeast referred to was made as follows. 30 g. of baker's yeast was crumbled and placed in a glass dish in an eight-inch vacuum desiccator over strong sulphuric acid. Within 24 hours it dried to a brittle mass, which was finely ground and replaced in the desiccator for a further 24 hours. It was then sterilised by grinding in pure *anhydrous* acetone two or three times for five minutes each time, and air dried. The resulting product was a light, dry powder which while apparently quite free from living cells showed considerable fermentative and respiratory activities. After washing a few times with cold distilled water it lost these powers, but they were restored on the addition of boiled extracts. This method, unlike the various German commercial methods, does not involve the use of very high pressures for the purpose of dehydrating the yeast, but it seems to be quite suitable for the preparation of small amounts for laboratory use. It is important to use carefully purified acetone, as quite small amounts of water, when mixed with it, have a markedly deleterious action on the enzymes.

The "acetoned" yeast was washed by placing 0·1 g. in each of four centrifuge tubes and adding 35 cc. of distilled water to each. The tubes were closed, well shaken, and allowed to stand for ten minutes. They were then shaken again, again allowed to stand for ten minutes, centrifuged, and the supernatant liquid decanted. This was done six times in all, except when otherwise stated.

The extracts were aerated and sterilised as before. The oxygen uptake was determined by the use of Barcroft micro-respirometers as previously described. The experiments performed in order to determine the presence or absence of the "alcoholic co-enzyme" were done in Barcroft apparatus with conical bottles similar to those used for blood-gas analysis. They can be filled with an inert gas if desired, but after a few trials this was found to be unnecessary. They were placed in a water-bath and shaken as in the respiration experiments. Some preliminary experiments showed p_H 5·8 to be the optimum,

so all solutions were adjusted accordingly before use. In each experiment of this type four instruments were used side by side, all being shaken simultaneously in the same water-bath. In each was placed 0·1 g. of the washed "acetoned" yeast.

In addition the instruments contained respectively:

Instrument	Glucose g.	Suspension fluid
A	·3	3 cc. of "stored" extract with 1 % KH_2PO_4 added, adjusted to p_H 5·8
B	None	" " " " " "
C	·3	3 cc. of "aerated" extract with 1 % KH_2PO_4 added; p_H 5·8
D	·3	3 cc. of 1 % KH_2PO_4 adjusted with NaOH to p_H 5 8

The results shown in the Tables I and II and in Figs. 1 and 2 are typical of a number of experiments.

Table I.

First respiration: The destruction of the "Respiration substance"							Second respiration: To test for the presence of the "Respiration substance"					
							Cmm. of O_2 absorbed per gram of muscle in one hour					
Extract	Vol. cc.	Muscle used	Weight grams	Times washed	Cc. of H_2O per gram of muscle for each washing	Time of aeration in hours	Phosphate solution	"Respired" extract	"Stored" extract	Muscle used	Times washed	Cc. of H_2O per gram of muscle for each washing	Temp. of respiration °C.
Rabbit muscle	50	Frog	5	6	50	4	34	94	266	Frog	8	50	21·5
Rat muscle	50	none	—	—	—	22	29	117	235	Rat	8	60	22·5
"	29	Rat	2·9	6	50	5	29	95	225	"	8	75	22·0
"	43	"	4·3	6	50	21	24	58	380	"	8	75	22·4
Rabbit muscle	80	Rabbit	8	4	40	17	28	59	213	"	8	75	22·2
"	72	"	7·2	4	40	18	39	76	511	"	8	75	22·0

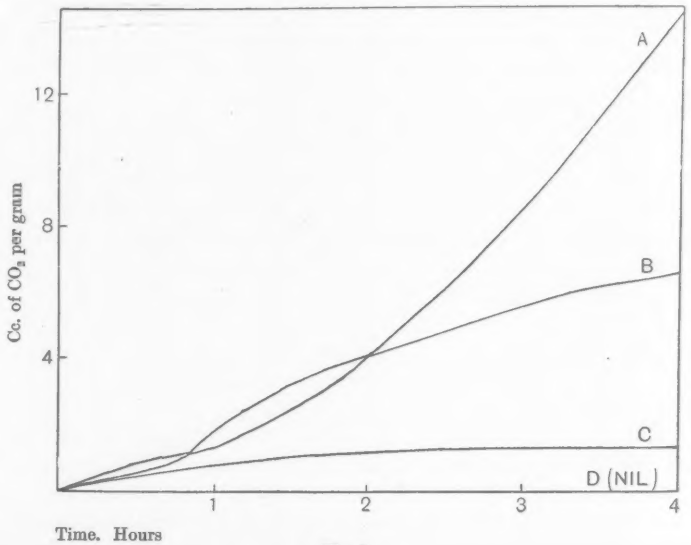

Fig. 1.

Table II. *The destruction of the alcoholic co-enzyme.*

No.	Extract	Time of aeration hours	Vol. of extract cc.	Substance added	Weight	Times washed	Cc. H_2O per g. each washing	Aeration p_H
1	Yeast	17	73	Rat muscle	7·3	4	40	7·6
2	Rabbit muscle	18	72	Rabbit muscle	7·2	4	40	7·6
3	Yeast	18	30	"Acetoned" yeast	0·6	—	—	5·8
4	Rabbit muscle	20	50	"	1	—	—	5·8
5	Yeast	21	50	Frog muscle	5	6	50	7·6

The test for the presence of the co-enzyme. cc. of CO_2 per g. of yeast.

No.	Temp. ° C.	Time in hours	A	B	C	D
1	22·0	4	14·56	6·65	1·34	0
2	22·0	4½	24·27	11·16	2·8	0·43
3	21·5	3½	28·3	2·62	3·55	0·06
4	24·0	4	7·32	4·86	0·97	0·27
5	21·1	4	12·1	—	1·25	0·14

The letters A–D refer to the four instruments, whose contents were as follow:

	Yeast g.	Glucose g.	Suspension fluid
A	0·1	0·3	3 cc. "stored" extract with 1 % KH_2PO_4 p_H 5·8
B	0·1	None	" " "
C	0·1	0·3	3 cc. "aerated" extract with 1 % KH_2PO_4 p_H 5·8
D	0·1	0·3	3 cc. 1 % KH_2PO_4 adjusted to p_H 5·8

The diagrams of experiments 1 and 2 only are published. On these diagrams, Nos. 1 and 2, the letters correspond to the letters in the above table.

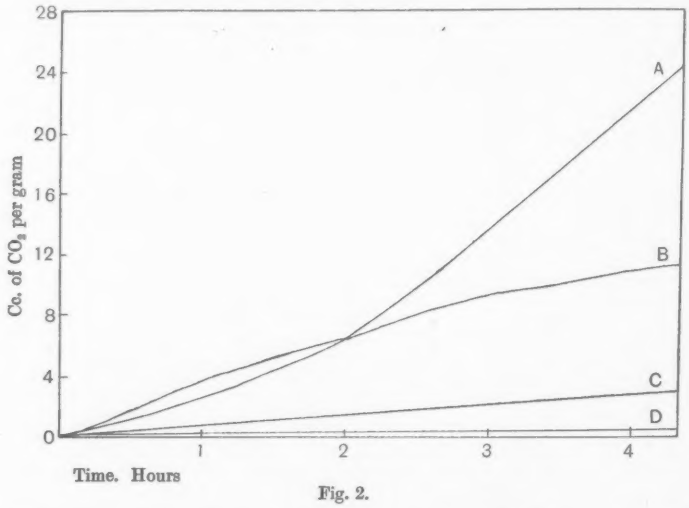

Fig. 2.

The "respiration substance" in muscle extracts, as in yeast extracts, is destroyed during respiration. The alcoholic co-enzyme in both yeast and muscle extracts is also destroyed by aeration in contact with washed muscle or with "acetoned" yeast. Yeasts from different sources differ in their power

of oxidising the alcoholic co-enzyme. Harden and Young [1907] showed that it is destroyed by yeast press-juice.

It may be mentioned that the fermentation co-enzyme in both yeast and muscle extracts is resistant to oxidation by air alone at ordinary temperatures, both at p_H 5·8 and 7·6. Little destruction can be observed after 48 hours of such treatment in some cases, and in no case was any marked destruction observed. The oxidation is dependent upon the presence of the washed tissue or the acetone-treated yeast cell.

Experiments were performed in order to determine whether either glutathione [Hopkins, 1921] or pancreatic insulin could function as the alcoholic co-enzyme in the presence of potassium phosphate. In both cases the results were negative. Glutathione showed a small stimulating effect on the oxygen uptake of the washed yeast. The insulin was wholly without effect. Nor could either oxidised or reduced glutathione restore the lost co-enzymic function to an extract which had been aerated with washed muscle. For a supply of active insulin I am indebted to Messrs Winter and Smith.

In view of Meyerhof's [1920, 2] opinion that the respiration of chopped, unwashed muscle is due to the oxidation of lactic acid formed during chopping, it was considered advisable to attempt to estimate the part played by lactic acid in the respiration of washed muscle in an extract. 0·5 g. of washed muscle was allowed to respire in 3 cc. of yeast extract and the oxygen uptake was measured. The lactic acid was estimated in some more of the same extract by Meyerhof's method [1920, 1].

The velocity of oxygen uptake was very much greater than could be obtained from pure solutions of lactic acid of similar concentrations, and in one experiment was greater than could be accounted for by the complete oxidation of all the lactic acid present. The results are given in Table III.

Table III. *Comparison between the lactic acid content of a cell extract and its power of stimulating the respiration of washed muscle.*

No.	Extract	Muscle	Times washed	O_2 per gram in phosphate	O_2 per gram 6 cc. extract	Temp. °C.	Time of respiration min.	R.Q.	Lactic acid mg. per cc.	Difference in cmm. between O_2 in extract and in phosphate at N.T.P.	Cmm. O_2 required to oxidise lactic acid in 6 cc. of extract
1	Yeast	Rat	4	29	777	22·7	60	—	·18	691	810
2	Yeast	Rat	4	61	1016	22·0	120	·92	·086	884	384
3	Yeast	Rat	4	55	1002	22·5	100	—	·23	875	1032
4	Rabbit muscle	Rat	4	38	368	22·5	80	·90	2·45	305	10992

Again when washed muscle or washed "acetoned" yeast was permitted to respire in a yeast or a muscle extract to which extra lactic acid had been added, the velocity of oxygen uptake was no greater than the sum of the velocities of oxygen uptake for the extract and for the lactic acid solution employed separately. Hence it would seem that there is in such extracts no substance capable of acting as a co-enzyme to the oxidation of lactic acid by washed muscle or yeast.

The results are set forth in Table IV and in Fig. 3.

Table IV. *The oxygen uptake of washed muscle and yeast respiring in cell extracts to which lactic acid had been added.*

			Respiration conditions						O_2 absorbed per gram in cmm.				
									X	Y	Z	W	
No.	Extract	Substance	Weight g.	Times washed	Cc. of H_2O per gram each washing	pH	Time min.	Temp. °C.	In 1% KH_2PO_4	In extract	In 0·5% lactic acid in 1% KH_2PO_4	In extract containing 0·5% lactic acid added	Calculated Y+Z−X
1	Yeast	Rat muscle	0·5	4	75	7·6	80	21·7	70	494	442	830	866
2	Yeast + 1% KH_2PO_4	"Acet." yeast	0·1	4	350	5·8	90	22·3	550	1790	2990	4290	4230
3	Rabbit muscle + 1% KH_2PO_4	"Acet." yeast	0·1	6	350	5·8	80	22·3	270	1640	2440	3260	3810

The values calculated in the last column are based on the assumption that the lactic acid is oxidised independently of the substances in the extracts. Fig. 3 shows the curves obtained in experiment No. 1, Table IV. The curves are lettered to correspond with the letters at the top of the columns.

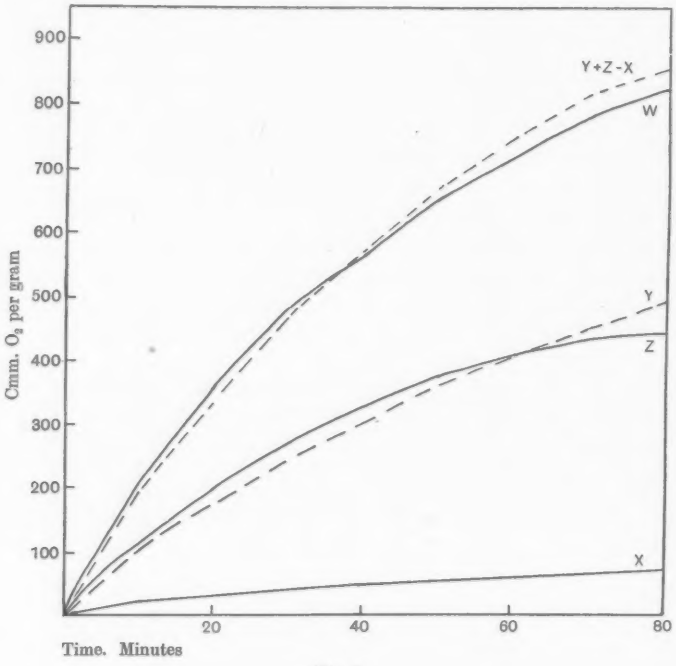

Fig. 3.

Discussion of Results.

The existing views on the respiration of such systems as chopped muscle and "acetoned" yeast may be stated as follows. When muscle is chopped, and suspended in buffer solution, the oxygen uptake is due solely to the

oxidation of the lactic acid present [Meyerhof, 1920, 2]. When washed chopped muscle or washed "acetoned" yeast is placed in tissue or yeast extract, the resulting respiration is due to the action of a "co-enzyme to respiration" in the extract catalysing the oxidation of oxidisable substances present, and hence providing a general stimulus to the cell respiration as a whole. This "co-enzyme to respiration" is identical with the alcoholic co-enzyme and is an oxygen carrier [Meyerhof, 1919, 2].

It seems to be tacitly assumed that the resting respiration of muscle is due almost entirely to the oxidation of the small quantity of lactic acid continually being formed and destroyed.

Certain facts may be noted. Muscle is known to possess several oxidative systems, each capable of dealing specifically with one type of substance, for instance the lactic acid, the succinic acid, and the glutathione systems. It has been shown that washed muscle is capable of oxidising various substances other than lactic acid without the aid of any oxygen carrier or other co-enzyme, which is soluble in water and therefore removable by washing; *e.g.* the sodium salts of lactic, succinic and citric acids are thus oxidised.

The glutathione system is an example of a true "co-enzyme to respiration" [Hopkins and Dixon, 1922]. Here the oxidisable substances are in the muscle residue. Under the conditions of the experiments described in this paper the oxygen consumption of this last system is small; moreover [Holden, 1923] it is possible to stimulate the respiration of washed muscle by yeast extracts from which glutathione has been removed by treatment with lead acetate.

Meyerhof [1919, 2] has himself shown that part at least of the "respiration substance" is precipitated by alcohol. Since lactic acid is not so precipitated, the fraction insoluble in alcohol must, from the standpoint taken in this paper, contain other oxidisable substances.

It will be observed that the claim based upon the work here described is that the stimulating effect of a tissue or yeast extract on the respiration of washed muscle may be attributed to the simultaneous oxidation of various substances present. The individual concentration of any one of these may be small, but collectively they may account for a considerable oxygen uptake. The existence of a co-enzyme is not disproved, but as yet it has not been shown to be necessary.

The experiments, in which extracts are used to which lactic acid has been added, show that there is no evidence of a soluble co-enzyme acting in the oxidation of this substance. Nor is the effect of an extract on the respiration of washed muscle due in any considerable degree to its lactic acid content.

One or more of the oxidisable substances in tissue and yeast extracts can function as the co-enzyme to alcoholic fermentation. It cannot be an oxygen carrier since it is capable of undergoing irreversible oxidation by washed muscle. It may be a hydrogen carrier of which the molecule is not dehydrogenated, but destructively oxidised during respiration. The fact that gluta-

thione, which can function as a hydrogen carrier, and which is present in yeast, cannot act as the alcoholic co-enzyme, cannot be considered as decisive evidence in view of the specificity of enzyme action.

The nature, the mode of action, and the significance in animal metabolism, of the alcoholic co-enzyme must still be regarded as obscure.

SUMMARY.

The "respiration substance" in an extract of mammalian muscle made with boiling water is destroyed by aeration in contact with washed rabbit, rat, or frog muscle.

Under similar conditions the alcoholic co-enzyme in yeast or muscle extracts is also destroyed, though it is not readily oxidised by air alone.

Neither glutathione nor pancreatic insulin can act as the alcoholic co-enzyme to washed "acetoned" yeast, in the presence of potassium phosphate, and at a suitable hydrogen ion concentration for the natural co-enzyme to act.

The "respiration substance" would seem to be in reality a collection of irreversibly oxidisable substances, though not, in the main, lactic acid.

A new method of making "acetoned" yeast is described.

I desire to express my gratitude to Prof. Hopkins for his kind advice and criticism. I also wish to record my indebtedness to the Department of Scientific and Industrial Research for a grant.

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LXXII. A COMPARISON OF CERTAIN OXIDISING ENZYMES OF THE HIGHER AND LOWER PLANTS.

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(*Received April 6th, 1924.*)

Peroxidase, the enzyme which induces the oxidation of guaiacum and other oxidisable substances by hydrogen peroxide, is known to be present in nearly all living plant cells. Many plants, moreover, are able to cause the blueing of guaiacum without the addition of hydrogen peroxide; these have been considered to contain a direct oxidase, phenolase or laccase. Bach and Chodat consider such an enzyme system to be composed of oxygenase in addition to peroxidase, oxygenase being described as an enzyme-like substance, which can unite with the oxygen of the air to form a peroxide, the system being analogous to that formed by hydrogen peroxide and peroxidase [Chodat, 1910]. The two components, oxygenase and peroxidase, have been separated to a certain extent by fractional precipitation with alcohol [Bach and Chodat, 1903], by dialysis, and by ultra filtration [Bach, 1916]. Most of the work of Bach and Chodat on oxidases has been carried out on the basidiomycetes, but oxygenase has been mentioned in connection with various phanerogams, *e.g. Rhus vernicifera*, the potato, the apple [Chodat, 1910], wheat and the sunflower [Bach and Oparin, 1923]. Onslow, M. W. [1920] has postulated that, for the higher plants, three components are present in what has been termed an oxidase or laccase, namely: an aromatic substance with two hydroxyl groups in the *ortho*-position, or "catechol" substance, from which a peroxide can be formed, and two enzymes; an oxygenase which catalyses the formation of peroxide from the "catechol" substance, and a peroxidase, which decomposes the peroxide with the production of active oxygen. The catechol substance is soluble in alcohol, therefore when an enzyme preparation is made by the usual method of alcoholic precipitation, it does not cause the direct blueing of guaiacum unless a substance containing the *ortho*-dihydroxy grouping (catechol, protocatechuic acid or aldehyde, caffeic acid, etc.) is added. The action of the oxygenase is apparent in the rapid darkening of the *ortho*-dihydroxy substance. It is, moreover, postulated that the discoloration on injury of plants containing a direct oxidase system is due to the action of the oxygenase on the catechol compound.

An enzyme solution prepared as described by Onslow [1920] oxidises *p*-cresol and causes quinol to darken, the products of the latter reaction, however, do not appear to blue guaiacum. Resorcinol and phloroglucinol are unattacked. Gallagher [1923] has denied that peroxide formation in the plant is due to enzyme action, and has stated that the so-called oxygenase, in the case of the potato, is in reality an autoxidisable lecithin-like substance which, on extraction from the plant, shows peroxide-forming properties. The ferric chloride reaction (catechol reaction) in the case of the mangold is affirmed to be caused by the presence of tannin, while the darkening of aqueous extracts of such plants as the potato and mangold is attributed to the action of tyrosinase on tyrosine, and not to the oxidation of a catechol derivative under the influence of oxygenase.

The experiments described by Onslow [1920] were repeated in the cases of the potato, apple, pear and banana, for plants showing the direct oxidase reaction, and the horse-radish and water melon, as peroxidase plants, the results described by the above investigator being corroborated. In addition to these, the artichoke and the groundsel, as oxidase plants, and the turnip, as a plant giving only the peroxidase reaction, were examined. In all cases where a direct oxidase reaction was present evidence was obtained for the existence of the two enzymes, oxygenase and peroxidase, and the presence of the "catechol" substance was indicated by the characteristic action of *o*-dihydroxy aromatic substances, namely a green reaction with ferric chloride in neutral solution, followed by a purple or red coloration on addition of sodium carbonate; moreover, the complete oxidase system could be synthesised by adding an extract of the aromatic constituents of the plant [Onslow, 1920] to an enzyme extract, prepared by alcohol precipitation, containing oxygenase and peroxidase, but no catechol substrate. In the case of the peroxidase plants there was no evidence for the presence of oxygenase, *i.e.* an enzyme extract did not catalyse the darkening of catechol derivatives with ensuing direct blueing of guaiacum, while no trace of the ferric chloride and sodium carbonate reaction was manifest.

In the case of the mangold, the oxygenase and peroxidase could again be demonstrated, while the presence of the catechol derivative, though indubitable, was sometimes difficult to show. Addition of an extract of the aromatic constituents of the plant to an enzyme extract prepared by alcohol precipitation caused, as in the cases cited above, the synthesis of the oxidase system, shown by the direct blueing of guaiacum.

The several mangold plants examined seemed to contain very different amounts (which were never very large) of this substance, so that in some cases the oxidase reaction was weak and an aromatic extract gave only a slight reaction with ferric chloride. The mangold appears to contain considerable quantities of a substance which is probably of pectic nature, being insoluble in strong alcohol, and precipitated by lead acetate. It seems possible that the catechol derivative is in combination with this substance. Pectic acid

itself, on hydrolysis with 1 % oxalic acid, under pressure, yields an insoluble acid which is probably galacturonic acid, analogous to glycuronic acid; this latter substance is known to combine with phenolic compounds. Attempts to hydrolyse the supposed pectic substance of the mangold with 1 % sulphuric acid, and to extract the catechol derivative with ether were unsuccessful.

It may be remarked, moreover, that the tannin reaction of an alcohol-ether extract of the mangold (*i.e.* a dark blue coloration with ferric chloride) obtained by Gallagher, was not always present.

The small content of the catechol derivative in the mangold may be attributed to the fact that this plant has a number of cultivated varieties, the chemical composition of which may well vary from that of the original type. Similar variations are shown in the potato; the variety King Edward, for example, gives a slight oxidase reaction and contains comparatively little of the catechol derivative.

The experiments of Gallagher [1923] in connection with an extractable lecithin-like substance were then repeated[1]. The phosphatide, after preparation and purification as described by the above author, was dissolved in alcohol, and, after standing for 19 days in an uncorked vessel, the alcoholic solution gave evidence of peroxide formation, on testing with horse-radish peroxidase and guaiacum. It is considered, however, that a reaction which requires so long a period to develop is hardly comparable with the instantaneous phenomena evident in the plant; there is, moreover, evidence that alcohol itself slowly undergoes peroxide formation on standing [Neuhaus, 1905; De Stoecklin, 1907]. Again, lecithin substances are present in peroxidase, as well as in oxidase plants, a phosphatide preparation from the turnip as an example from the former class, made exactly as that described in the case of the potato, showed similar peroxide forming properties.

The oxidase system of the fungus *Lactarius vellereus*, studied by Bach and Chodat, and others, was then investigated. Preparations of a crude enzyme extract were made according to the method described by Onslow [1920] which had been used for the phanerogams, namely, the plant tissue was rapidly and thoroughly pounded with 97 % alcohol and was repeatedly filtered to dryness on a Buchner funnel; the residue was then extracted with water for about 15 minutes, and filtered, the filtrate containing a crude solution of the enzymes present. Unlike a similar enzyme preparation from the potato or other phanerogam, this water extract contains a complete direct oxidase system, that is, it is able to blue guaiacum without the addition of H_2O_2. If, therefore, any substrate comparable to the catechol derivative of the higher plants exists, this substance does not seem to be soluble in alcohol. Thorough extraction of the plant tissue with acetone, ethyl acetate, benzene

[1] An alcoholic extract of potatoes, prepared as described, was concentrated *in vacuo*; the residue was then divided into two portions, one half being extracted with ether, and the other with chloroform, the latter procedure providing a control for the more rapid peroxide-forming properties of the ether itself.

and ether failed to disintegrate the oxidase system. The oxidases of the basidiomycetes seem thus to be fundamentally different from those of the higher plants. The behaviour of the phenolases of the fungi *Lepiota Prossera*, *Agaricus campestris* and *Russula fragilis* appeared to be similar to that of *Lactarius vellereus*.

Further examination of *Lactarius* showed that an enzyme extract prepared as above oxidises tyrosine and *p*-cresol and causes the phenols, quinol, resorcinol and phloroglucinol, to darken. The action on catechol is extremely rapid, suggesting the existence of some system analogous to the oxygenase described by Onslow for the phanerogams.

The latex of *Lacterius vellereus* does not darken on injury of the plant; moreover, extraction with hot alcohol, as described by Onslow [1920], of both this fungus and *Agaricus campestris*, failed to reveal any evidence of the presence of an *o*-dihydroxy aromatic compound. These differences between the oxidase systems of the higher and lower plants may explain the existence of the different conceptions of the nature of the oxygenase postulated by Bach and Chodat and by Onslow. Fraenckel [1922], in accordance with the theory of the former authors, explains the disintegration of the oxidase system of the higher plants by assuming that, in this case, the oxygenases are much more susceptible to the toxic action of alcohol than those of the basidiomycetes.

Similarly, the various conflicting theories on the nature of tyrosinase may be explicable if this enzyme shows corresponding differences in the phanerogams and the cryptogams. The observations which have been made on this subject are difficult to correlate and to interpret.

Chodat [1910] considers tyrosinase to be a single enzyme which oxidises tyrosine, *p*-cresol, and certain other substances when in the presence of the latter compound. He has prepared a tyrosinase free from both phenolase and peroxidase from the fungi *Lactarius vellereus* and *Russula foetens*. Chodat and Staub [1907] prepared an oxidase-free tyrosinase from potato peelings[1]; they found that this enzyme brings about the deamination of amino-acids, resulting in the formation of aldehydes. Schweizer [1917] suggests that deamination caused by tyrosinase is due to its activity as an oxidising enzyme, as deaminases, although their existence has been long assumed, have never been isolated. He remarks, moreover, that laccase is an improbable constituent of tyrosinase, as the two enzymes can work in different reaction media.

Bertrand [1886] prepared a laccase-free tyrosinase from *Russula*, and Bertrand and Muttermilch [1907] made from wheat bran a tyrosinase which gave none of the reactions of laccase.

Bach [1914] on the contrary, has been unable to prepare tyrosinase which does not contain both phenolase and peroxidase either from the fungi or from

[1] Tyrosinase prepared by alcohol precipitation from the phanerogams does not give the guaiacum reaction for laccase (*i.e.* blueing of guaiacum without the addition of H_2O_2) as the "catechol" substrate for the oxygenase is removed by alcohol.

the potato. He attributes the inactivity towards oxidase reagents of the enzymes obtained by Chodat and his pupils to an alteration of salt content. He considers, moreover, that in the preparation from wheat bran obtained by Bertrand and Muttermilch the oxygenase was partly destroyed (see footnote, p. 546) and found that acceleration of the oxidation of tyrosine was brought about by addition of hydrogen peroxide. Bach therefore considers that tyrosinase is a mixture of two enzymes, a deaminase and a phenolase.

Onslow [1923] in studies of the phanerogams, using crude enzyme extracts, could find no plant without phenolase if tyrosinase were present. In certain plants, however, which contained a well-marked oxidase, no tyrosinase could be demonstrated (*Urtica dioica*, *Nicotiana tabacum*, etc.).

Dodge [1919] considers that fungus tyrosinase has no deaminase action, as ammonia estimations made during the course of the reaction with tyrosinase bore no relationship to the disappearance of amino nitrogen, which was investigated concurrently. He attributes the ammonia found by Chodat and Schweizer to the activity of a deaminase precipitated with tyrosinase from the plant extracts used. This author cites the work of Beyerinck [1913] in support of the theory that tyrosinase contains two component enzymes.

The possibilities as to the nature of tyrosinase seem therefore to be as follows:

1. Laccase and tyrosinase may be two separate unassociated enzymes, as found by Chodat. As Bach and Onslow have found, however, that tyrosinase is always accompanied by laccase, the latter enzyme may always be a component of the former.

2. The two classes of reactions may be due to the same enzyme—to which special names have been applied when the reaction substrates and products are different. The inactivity towards tyrosine of certain oxidase preparations and of hydrogen peroxide and peroxidase may be due to the inadequate salt content of the medium.

It has been mentioned above that Gallagher [1923] attributes the darkening of the mangold to tyrosinase rather than to the action of oxygenase on a catechol derivative. This may seem possible for such a plant as the mangold where the amount of the catechol substance is small and the oxidase itself is feeble, but such an interpretation appears to be inadequate in a case such as that of the apple, where the presence of tyrosinase cannot invariably be demonstrated, possibly owing to the high concentration of acid.

The oxidation of *p*-cresol, with the production of a deep orange colour, is held by Chodat [1907] to be a specific reaction for tyrosinase, the oxidase or peroxide-peroxidase system giving a colourless substance insoluble in water, with this reagent. Onslow [1923] from work on the higher plants has found, however, that enzyme extracts from all the oxidase plants examined oxidise *p*-cresol, the products of the reaction being able to blue guaiacum. *p*-Cresol in such cases acts analogously to a catechol derivative, that is, as a substrate for oxygenase, rather than as a reagent for "active" oxygen. Onslow has

suggested that an *o*-dihydroxy-derivative may be formed from *p*-cresol by "active" oxygen or that traces of such a dihydroxy-derivative may be present as an impurity in the substance itself. As, however, the characteristic orange colour does not always appear in the absence of tyrosinase, it seems possible that a specific oxidising enzyme may be necessary to act on *para*-hydroxy-compounds. Further work on the action of oxidases on such substances might give indications of the relationship of laccase with the oxidising constituent of tyrosinase.

Summary.

1. A fundamental difference appears to exist between the oxidases of the higher plants and those of the basidiomycetes, in that those of the former which have been examined consist of three components, a catechol derivative and two enzymes, oxygenase and peroxidase, while the oxidase of the basidiomycetes has so far been separated into two constituents only, an enzyme-like peroxide and peroxidase.

2. The experiments of Onslow [1920] on certain phanerogam oxidases have been repeated and the results confirmed. The work of Gallagher [1923] on extractable plant phosphatides has been repeated; its interpretation as regards the oxidase reaction is discussed in the text.

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LXXIII. OXIDISING ENZYMES. VII. THE OXYGENASE OF THE HIGHER PLANTS.

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In a recent contribution to this *Journal*, Gallagher [1923] states that there is no definite evidence for the existence, in the higher plants, of an enzyme (oxygenase) which catalyses the formation of peroxide. The quick direct blueing of guaiacum, which is characteristic of the tissues and extracts of a number of plants, is stated to be due to the presence of an autoxidisable lecithin-like substance; such a substance has been extracted from two sources, namely, the potato tuber and the mangold root.

In criticism of the above statements, it may be mentioned that the presence of a thermolabile catalyst, which catalyses the autoxidation of catechol, protocatechuic acid, protocatechuic aldehyde and other catechol derivatives with the production of peroxide, can be readily demonstrated in the potato tuber [Onslow, 1920] and in the mangold root [Robinson, 1924] as well as in many other plants. These enzymes also act on catechol derivatives present in the plant; they are, moreover, absent from plants of which the tissues show no direct blueing.

The denial of the existence of an enzyme catalysing the formation of peroxide, and the substitution of another system consisting of an autoxidisable lecithin-like substance as the cause of the rapid blueing of guaiacum is open to further criticism. First, the crude autoxidisable lecithin-like substance can be obtained equally well from the tissue of a plant, for example, the turnip root, which shows no direct blueing of guaiacum [Robinson, 1924]. Secondly, the lecithin-like substance needs a considerable time (from 5–19 days) to autoxidise before giving any blueing with guaiacum in presence of peroxidase, whereas in the plant tissues the reaction is usually almost instantaneous. Thirdly, very many substances have this power of autoxidation, and, after a longer or shorter period, are able to function as peroxides. Lecithin-like compounds are not peculiar in this respect; the property is shown, for instance, by aldehydes (formic, acetic[1], valeric, benzoic, salicylic, cinnamic), caprylic alcohol, oleic acid, pyruvic acid, many terpenes, and, in fact, by most plant extracts on standing in air for any length of time.

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[1] Samples of acetaldehyde (Merck, Kahlbaum) were found to function not only as peroxidases (blueing of guaiacum in presence of hydrogen peroxide) as stated by Gallagher [1924], but also at the same time as oxidases (direct blueing of guaiacum); others again as peroxides (blueing of guaiacum in presence of peroxidase).

LXXIV. THE OCCURRENCE OF NUCLEIC ACID DERIVATIVES IN NITROGEN-FIXING BACTERIA.

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The occurrence of nucleic acid in the cells of the tubercle bacillus is a well-known fact, for Ruppel [1898] separated an acid under the name of tuberculinic acid, which has been shown [Levene, 1901, 1904; Johnson and Brown, 1922, 1, 2], to have all the properties of a nucleic acid, and which apparently constitutes from 2·0 to 3·5 % of the weight of the organism [Long, 1920]. Very little work has been done with regard to the appearance of this acid in other bacteria, but attention was directed to the possibility of its occurrence in the nitrogen-fixing bacteria by the fact that the products of these bacteria have a growth-promoting effect on plants [Bottomley, 1917], an effect which is shared by yeast [Mockeridge, 1924] and by extracts of soils and manurial materials which all contain nucleic acid or its derivatives [Bottomley, 1919; Mockeridge, 1920]. In view of the constant occurrence of these substances in all other materials which are known to have this growth-promoting effect on plants, it was decided to make a preliminary investigation of *Azotobacter chroococcum* in order to determine whether it contained any nucleic acid or derived substance. For this purpose as large a quantity of the organism as possible was required, and it was obtained by growth on mannitol agar. A medium consisting of nutrient materials in the following proportion was prepared:

Mannitol	1 g.	Calcium carbonate	0·2 g.
Dipotassium phosphate	0·2 g.	Agar-agar	2 g.
Magnesium sulphate	0·02 g.	Distilled water	100 cc.

This nutrient agar was poured into six-inch Petri dishes, which were then sterilised in an autoclave at a temperature of 120° for half-an-hour. When cool and solidified, the medium was inoculated with a suspension in sterile distilled water of *Azotobacter* from a colony of recent isolation from soil. The plates were then incubated at a temperature of 26° for about 14 days, at the end of which period the surface of the medium was generally covered with a mass of the bacteria of the typical coccus form, embedded in wide slime capsules often several times their own diameter, forming large masses of mucilaginous material which could be readily scraped off the surface of the agar and could thus be obtained free from nutrient materials. The available

incubating space was utilised many times over in this way, in order to obtain sufficient material for examination, and several successive fresh isolations of *Azotobacter* from soil were made for the purpose, since it was always found that the organism grew most rapidly when only recently isolated.

The thick gelatinous culture thus obtained was examined, in the first place, in three different ways. One portion was sterilised in the autoclave at a temperature of 140°, and then extracted with a 10 % solution of caustic soda. A second portion was extracted at once, without previous sterilisation, with a similar caustic soda solution, and the third portion was merely shaken with distilled water. This last method has so far given negative results, but it is proposed to repeat it on the much larger scale which is obviously required in order to extract the small quantities in which these materials occur in the slime capsule, if they are to be found at all.

Except for the fact that the first portion of the bacterial material received the preliminary treatment of being heated in the autoclave to a temperature of 140° for two successive periods of one hour each, separated by a two hours' interval, the remaining two portions were treated similarly. The gelatinous material, autoclaved or otherwise, was covered with a very large excess of 10 % caustic soda solution, placed in a stoppered bottle and shaken in a shaking machine for about 24 hours, in order thoroughly to extract the required materials. It was then almost neutralised with hydrochloric acid, and allowed to stand for a few hours, in order that the bacteria might settle out as much as possible. The supernatant liquid was decanted off and centrifuged in order to get rid of the remainder of the bacteria, and as a last resort the clear liquid was passed through a Berkefeldt filter under pressure. Microscopic examination proved that this liquid was free from bacteria, so it was acidified with acetic acid in order to precipitate any protein. The slight precipitate was removed by filtration, the whole filtrate just neutralised with caustic soda, and concentrated *in vacuo* to a small volume. Concentrated hydrochloric acid was added in sufficient quantity to render the liquid strongly acid, and the whole poured into about five times its total volume of absolute alcohol. A slight flocculent precipitate separated out on long standing, and this was removed by filtration. From the method of preparation it was probably nucleic acid, but the quantity was too small for further examination.

The filtrate from this precipitate was accordingly further examined for nucleic acid derivatives. For this purpose it was just neutralised with caustic soda, and concentrated *in vacuo* to a small bulk in order to remove the alcohol. A small portion of the liquid was tested for the presence of phosphoric acid by both magnesia mixture and ammonium molybdate, with positive results in both cases. A further portion was submitted to Molisch's test, which proved the presence of carbohydrates, and that these were reducing sugars was shown by a slight reduction of Fehling's solution. The liquid also responded to the phloroglucinol and orcinol tests for pentoses, so that part, at least, of the carbohydrates present consisted of these sugars.

Separation of the Purine Bases.

The remainder of the liquid was used for the separation of the purine and pyrimidine bases, the methods followed being those of Jones [1914], with slight modifications. On account of the large amount of chloride present, the use of a silver salt was avoided, the purine compounds being precipitated by the use of a copper salt, while an attempt was made to separate the pyrimidine derivatives by the use of a mercuric salt. In order to accomplish the desired results, the liquid was brought to the boil, and concentrated ammonia added, the whole being kept briskly boiling all the time, until no further precipitate formed. This precipitate would contain all the phosphoric acid, together with guanine, if present. The liquid was boiled for a short time after the addition of the ammonia, and the precipitate was then removed by filtration, washed with dilute ammonia several times, and the filtrate and washings added together. The precipitate was warmed with dilute caustic soda solution in order to dissolve any guanine present, while the phosphoric acid remained unaffected. This insoluble material was filtered off, and to the filtrate was added dilute acetic acid, in order to precipitate any guanine. An appreciable precipitate was obtained, which was filtered off, washed with dilute acetic acid, and submitted to various tests indicated below in order to identify the material.

The combined filtrate and washings from the guanine and phosphoric acid precipitate were acidified with dilute sulphuric acid. Crystalline copper sulphate was added in large excess, and the liquid brought to the boil; the addition of a saturated solution of sodium bisulphite then resulted in the formation of a white precipitate. This reagent was therefore continually added in successive small quantities until the precipitate formed began to show a reddish tinge. Boiling was then continued rapidly for a short time, and the white precipitate of copper-purine filtered off, thoroughly washed with boiling water, suspended finally in hot water and decomposed with sulphuretted hydrogen. The black precipitate of copper sulphide was removed by filtration, using a filter pump to accelerate the process, washed with water, and the combined filtrate and washings concentrated *in vacuo*, filtered, and used for the preparation of salts of adenine for purposes of identification.

Separation of Pyrimidine Derivatives.

The filtrate from the copper-purine compound was then examined for the presence of pyrimidine bases. The liquid was first treated with sulphuretted hydrogen, the precipitate of copper sulphide filtered off, and the sulphuric acid removed with baryta. The filtrate was concentrated somewhat, after which a solution of mercuric sulphate was added and the whole acidified with sulphuric acid. After standing for 24 hours the very slight precipitate was filtered off, washed with water slightly acidified with sulphuric acid, suspended in hot water and decomposed with sulphuretted hydrogen. The precipitated mercuric sulphide was removed, and the filtrate concentrated *in vacuo*.

An attempt was made to precipitate cytosine in form of the picrate by the addition of a hot saturated solution of picric acid. After standing for

24 hours no precipitate had appeared, so the liquid was freed from picric acid by shaking in a separating funnel with sulphuric acid and ether, after which the sulphuric acid was removed with baryta. The filtrate from the barium sulphate was again concentrated, and set aside in order to allow of the separation of any uracil present. After some days no deposit had appeared, so the liquid was subjected to the colour test of Wheeler and Johnson, with positive results. Apparently, therefore, the pyrimidine derivatives, though present, occurred in such small quantity as to fail to separate out. Whether they do actually occur in less quantity than the purines, or whether they had been lost during the process of separation, is a matter for future investigation.

Examination of the Purine Bases.

Various tests were then carried out in order to identify the two materials separated. A small portion of the precipitate which was suspected to be guanine was warmed on porcelain with a drop of nitric acid. When evaporated to dryness it left a muddy yellow spot which became brownish-red on the addition of sodium hydroxide: a reaction which indicated the presence of guanine. A small portion of the precipitate was treated with hot sulphuric acid and the remainder was dissolved in hydrochloric acid, and the latter solution allowed to stand for a considerable time in a desiccator. A mixture of needle-shaped and long tetrahedral crystals of the hydrochloride was thus obtained eventually, and from this chloride were prepared the picrate, by the addition of picric acid to the solution, and the dichromate, by the similar addition of a solution of chromic acid. The former salt separated out as a mass of long, fine, thread-like needles, which had a woolly appearance in bulk, and which dried to a felt-like mass. On heating, this salt became orange-red, and finally decomposed without melting at 190°. The latter salt appeared very slowly in the form of truncated prisms, bright orange in colour, which changed to a dark violet when the salt was heated to 100°. The solution in hot sulphuric acid was cooled rapidly, when the sulphate separated out in the form of needle-shaped crystals. The formation of these salts, together with the method of preparation of the material, the fact that it formed a gelatinous precipitate with ammoniacal silver nitrate, dissolved in hydrochloric acid and was thrown out of solution by ammonia, afforded sufficient evidence to identify the substance as guanine.

The material which was separated out by precipitation with copper sulphate and sodium bisulphite, and which was in all probability adenine, was tested first of all for the formation of a picrate. To a portion of the liquid, picric acid in saturated solution was added, and a very little ammonia, when a precipitate consisting of clusters of fine needles was at once obtained. When redissolved and recrystallised this appeared in the form of somewhat elongated prisms which were dried and found to melt with decomposition at about 277°, the approximate melting point of adenine picrate. Another portion of the material was tested with ammoniacal silver nitrate, when a gelatinous precipitate appeared; to yet another was applied Kossel's test for purines,

which gave a positive result; a drop of ferric chloride was added to a fourth portion, resulting in a deep red colour, unchanged by heating. From the remainder of the liquid salts were prepared; the hydrochloride, by the addition of hydrochloric acid, separating in flat, deliquescent prisms; the dichromate, by the addition of chromic acid, forming six-sided plates; and the double salt with gold chloride, occurring as long, orange-coloured prisms. All these reactions confirmed the material as adenine.

Discussion.

Exactly similar materials were isolated from the *Azotobacter* growth, whether the organisms had been autoclaved or not, the only difference being that in the case of the autoclaved material a bigger yield of the organic bases was obtained. It is clear, therefore, that whether or not nucleic acid occurs as such within the *Azotobacter* cells, there are elaborated within the organisms all the essential radicles for its formation, viz.: phosphoric acid, a carbohydrate, two purine bases, adenine and guanine, and also pyrimidine bases.

Apart from any bearing that this result of the work may have upon the question of auximones, it is of interest from the point of view of the actual organisation of the bacteria themselves, for the fact that there are to be extracted from these cells substances which must be regarded as essentially bound up with a nucleus is a strong point in favour of the occurrence within the bacteria of at least the rudiments of a nucleus. It is intended to carry out quantitative work in the hope of obtaining some idea of the extent to which actual nuclear material can be regarded as being present in these lowly organisms. It is also hoped to identify the pyrimidine derivatives present, and so to ascertain whether this organism is similar to the tubercle bacillus in the occurrence of thymine [Johnson and Brown, 1922].

From the point of view of the metabolism of these bacteria it is of interest to note that these relatively complex nitrogenous substances have been synthesised by the organisms from elementary nitrogen, since the bacteria are cultivated in a medium absolutely free from combined nitrogen, their only source of this element being the atmosphere. A similar investigation of other nitrogen-fixing bacteria will be made in due course.

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LXXV. CALCIUM CONTENT OF THE BLOOD DURING PREGNANCY. PART II.

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(*Received April 9th, 1924.*)

A previous investigation gave the results of a study of the calcium content of the blood during normal cases of pregnancy [Widdows, 1923].

That work has been extended to cases other than normal, and the present paper records results obtained where previous pregnancies had shown departures from the normal.

The earlier work had established the following two points. First, that the very heavy demand that undoubtedly is made by the foetus for calcium at certain stages of its development is met by a very perfectly balanced calcium metabolism as at no time is the variation of the calcium content of the serum very marked. Second, that in the later stages of pregnancy there is a distinct tendency for the calcium content of the blood to decrease, and to increase directly after confinement with a recovery to normal, with further increase during the early stages of lactation.

While working on the normal cases a certain number showing previously abnormalities such as miscarriages, stillbirths, etc. were investigated, but no definite relationship was detected between the abnormality and the calcium content of the blood.

Having established the normal variations in the calcium content of the blood, it was thought that a systematic study of these cases of abnormal pregnancies, on the same lines as those followed before, might lead to some more definite results.

A large number of such cases have now been investigated, and as in the earlier work particular attention has been paid to those periods at which an abrupt change in the calcium metabolism of the foetus seems to occur. The samples of blood for analysis were taken between the 3rd and 4th months, the 6th and 7th months, and the 8th and 9th months, and they were taken from the same individual at these different periods. The analyses were made on the serum in all cases, and the procedure was the same as that detailed in the earlier paper.

The cases studied had shown in earlier pregnancies miscarriages, stillbirths, excessive vomiting during the later stages of pregnancy, albuminuria, eclampsia.

The amount of calcium is always given in milligrams per 10 cc. of serum. The average calcium per 10 cc. of serum is 0·99 milligram.

Table I is a reproduction of the table showing the results for the normal cases.

Table I.

Stage	Number of specimens analysed	Number of cases investigated	Average calcium found
3–4 months	23	22	1·11
6–7 „	27	24	0·996
8–9 „	25	24	0·94
Post-confinement within 14 days	18	18	0·994

In Table II the figures are the mean for the different periods of all cases, showing the above abnormalities, without classification.

Table II.

Stage	Number of specimens analysed	Number of cases investigated	Average calcium found	Range of calcium found	
				Highest	Lowest
3–4 months	39	36	1·07	1·2	0·91
6–7 „	82	72	1·00	1·2	0·71
8–9 „	86	77	1·00	1·14	0·79
Post-confinement within 14 days	68	67	0·988	1·17	0·72
3–4 months	47	44	1·03	1·13	0·73

The above numbers show a drop in the calcium content after the 4th month of pregnancy. From the 6th till the 9th month however it remains almost constant, whereas in normal cases there was a distinct drop during the 8th–9th month. There is the same tendency to rise after confinement as in the normal cases.

The cases in this table have been analysed and arranged in groups, and as far as possible those cases collected together which are characterised by only one abnormal factor. The results are shown in Tables III, IV, V, etc.

In Table III the results are drawn from cases showing anyone or all of the following characteristics: stillbirths, miscarriages, and premature births.

Table III.

Stage	Number of specimens analysed	Number of cases investigated	Average calcium found	Range of calcium found	
				Highest	Lowest
3–4 months	23	20	1·09	1·2	0·98
6–7 „	43	40	0·998	1·14	0·71
8–9 „	34	33	0·993	1·14	0·79
Post-confinement within 14 days	37	37	1·00	1·17	0·75
3–4 months	19	19	1·04	1·13	0·95

These results are similar to those shown in Table II.

In Table IV cases have been collected showing miscarriages as their only abnormality.

Table IV.

Stage	Number of specimens analysed	Number of cases investigated	Average calcium found	Range of calcium found	
				Highest	Lowest
3–4 months	19	15	1·10	1·2	0·99
6–7 ,,	19	18	1·02	1·16	0·99
8–9 ,,	10	10	1·02	1·14	0·94
Post-confinement within 14 days	13	13	1·00	1·17	0·81
3–4 months	8	8	1·03	1·07	0·97

The striking point about the above numbers is the very slight variation of the calcium value from the 6th to the 9th month. Also the calcium content of the blood throughout the pregnancy is high.

In two cases the calcium of the urine has been estimated, and in both cases it has been found to be very high. In one case the total output of calcium in the form of calcium oxide per day was 0·44 g. and in the other 0·847 g. per day. The normal output of calcium in the form of calcium oxide varies from 0·2 to 0·35 g. per day.

In Table V the results are taken from cases where stillbirths have occurred.

Table V.

Stage	Number of specimens analysed	Number of cases investigated	Average calcium found	Range of calcium found	
				Highest	Lowest
3–4 months	2	2	1·01	0·996	0·98
6–7 ,,	10	10	0·995	1·14	0·95
8–9 ,,	11	11	0·96	1·1	0·75
Post-confinement within 14 days	8	8	0·994	1·05	0·75
3–4 months	3	3	1·07	1·11	1·07

The above table is, on the whole, in accordance with the results obtained in the wholly normal cases, *i.e.* a distinct fall in the calcium during the last months of pregnancy and an immediate rise after confinement.

Tables VI, VII, VIII, deal with cases showing excessive vomiting during the last months of pregnancy, albuminuria, and eclampsia. The number of cases showing albuminuria and eclampsia are not large and cases are still being studied, but it was thought in connection with the other results it might be interesting to record the observations already made.

In Table VI are recorded the results from cases showing excessive vomiting during the later stages of pregnancy.

Table VI.

Stage	Number of specimens analysed	Number of cases investigated	Average calcium found	Range of calcium found	
				Highest	Lowest
3–4 months	4	4	1·07	1·14	1·01
6–7 ,,	13	13	1·06	1·2	0·89
8–9 ,,	16	16	1·02	1·07	0·96
Post-confinement within 14 days	13	13	0·94	1·05	0·73
3–4 months	8	8	1·05	1·11	0·97

In all the above cases the calcium of the blood throughout pregnancy is definitely above the average, as in the case of miscarriage only as the abnormality. During the last month the calcium value tends to decrease but still remains high and immediately after confinement it decreases still further to rise again during the early months of lactation.

The results from cases showing albuminuria are found in Table VII.

Table VII.

Stage	Number of specimens analysed	Number of cases investigated	Average calcium found	Range of calcium found	
				Highest	Lowest
3–4 months	0	0	—	—	—
6–7 ,,	2	2	0·97	1·04	0·89
8–9 ,,	11	9	0·95	1·08	0·79
Post-confinement within 14 days	6	6	0·93	1·00	0·84
3–4 months	9	9	1·03	1·08	0·999

In these cases of albuminuria the calcium content of the blood is below normal value during pregnancy, decreases during this time and just after confinement, rising again during the early months after confinement.

Table VIII gives the results obtained from cases showing eclampsia or pre-eclamptic symptoms.

Table VIII.

Stage	Number of specimens analysed	Number of cases investigated	Average calcium found	Range of calcium found	
				Highest	Lowest
3–4 months	3	3	1·05	—	—
6–7 ,,	4	4	1·03	1·08	0·94
8–9 ,,	4	4	1·05	1·14	0·96
Post-confinement within 14 days	5	5	0·89	1·04	0·73
3–4 months	1	1	1·05	—	—

Here again the calcium value is high throughout the pregnancy and shows no decrease during the last months. Immediately after the confinement however there is a considerable decrease, the value rising again during the early months after confinement.

These results certainly strengthen one of the conclusions drawn from a study of normal cases; viz. that although at certain stages of its development the foetus makes a very heavy demand for calcium, yet this demand is met by a very perfectly balanced calcium metabolism, because at no time during the pregnancy, even in these abnormal cases, is the variation of the calcium very marked. The most marked change is shown in the few cases of eclampsia which have been studied, where the calcium value drops from 1·05 to 0·89 immediately after confinement.

The values for the blood calcium obtained at the different periods in certain of these abnormal cases are in accordance with those of the normal; many however show a distinct variation.

The results from the cases of stillbirths show the normal variation.

The cases of excessive vomiting and albuminuria show the normal variation up to the 9th month, but a fall directly after confinement, rising again after 3–4 months.

The calcium, throughout the pregnancy, is above the average in the cases of excessive vomiting, but below in those of albuminuria. The cases of miscarriage and eclampsia show also, throughout the pregnancy, a calcium content above the average, but very constant during the last months. There is no decrease between the 8th and 9th months, but a distinct drop immediately after confinement, rising again after 3–4 months.

It has been suggested that the high calcium content of the blood of women in labour is due to a tendency of the hydrogen ion concentration of the blood at a fixed carbon dioxide tension to be increased, the severe muscular effort causing lactic acid to be passed into the blood stream.

In those cases of eclampsia, excessive vomiting and miscarriages the high calcium value might be attributed to the same cause, an increased acidity of the blood. Cook [1924] does find in certain cases of eclampsia a definite fall in the plasma bicarbonate, but attributes this to the effect of the convulsions and the labour itself, and states that it is generally agreed that the acidosis factor plays a negligible part in the "toxaemias" of pregnancy. This is supported by the work of Marrack and Bone [1923].

What connection there is, if any, between these calcium values and the abnormalities studied it is difficult to say. The calcium variation of the blood can be only one of the many influencing factors. Yet the above numbers do show in certain cases a calcium value definitely above the average for blood and in other cases definitely below.

If it is possible to correct this variation from the normal easily, one of the factors that may be a disturbing influence in these particular cases will be eliminated and the study simplified.

J. B. S. Haldane has made experiments showing how the calcium content of the blood can be largely increased or decreased, but the methods employed are too drastic for ordinary patient treatment.

If by a variation of the diet the calcium content of the blood could be appreciably affected this would be the most pleasant method of correction. Since there is always a large reserve of calcium in the bones, and large amounts of calcium are excreted in the urine and faeces, the small variations that occur in the blood calcium are probably regulated by the mechanism in the body for the elaboration of calcium rather than by the quantity of calcium available in the system.

Vines [1921] in his work on the relationship between the ionic and combined calcium of the blood has worked at the subject from this point of view, and his suggested parathyroid treatment may regulate not only the above relationship, which he considers all important, but also the actual total normal amount of calcium.

With regard to the possibility of altering the calcium value by diet, the evidence seems to be very contradictory.

Halverson and Bergeim [1917], Neurath [1911], Katzenellenbogen [1913], Denis and Minot [1920] and Clark [1920] from their experiments conclude that the blood calcium is little affected by the addition of calcium to the diet, and that in any case it is not increased above the normal. Meigs, Blatherwick, and Cary [1919] from their work on cattle come to the same conclusion.

On the other hand, Voorhoeve [1911] found that he could increase the calcium value provided a sufficiently large amount of calcium was added to the food per day. He gave 2·7 g. of calcium oxide per day for several days. Clark however thinks his methods of analysis are open to criticism. Boggs [1908] has also shown that when the calcium content of the blood is low it can be increased by the administration of calcium lactate or acetate. In the case of a haemophylliac [Widdows, 1923] it was found that by the administration of calcium chloride the calcium value of the blood increased from 10·5 to 11·2 mg. per 100 cc. of blood.

These results suggest that when the calcium value is low, by the addition of calcium salts to the diet, the value may be raised to normal. On the other hand, since Halverson and Mohler suggest that under ordinary conditions the plasma calcium is kept nearly constant by means of the enormous calcium reserve in the bones, in the case of a pregnant woman, when the blood calcium is high, a starvation calcium diet might have serious results.

As suggested above it is probable in these abnormal cases the high or low calcium values are only symptomatic of some more deeply seated disturbance involving the calcium metabolism, and that, although in normal cases of pregnancy a diet rich in calcium is of importance to help to counteract the great demand of the foetus for this element in the later stages of its growth, in these abnormal cases there can be no effective treatment until the real cause of the disturbance is discovered.

Table IX has been included in this paper, as it was thought the figures recorded might be of interest in view of Blair Bell's work [1908]. The latter's work suggested that just before the birth of the child the calcium of the blood of the mother should rise, and fall immediately after confinement. During last year it was possible to examine the blood of a few cases under these conditions, and the figures certainly are in agreement with Blair Bell's theory.

Table IX.

Case	Calcium about 8 months	Days before pregnancy	Calcium found	Days after pregnancy	Calcium found
A	0·94	2	0·99	20	0·94
B	0·97	1	1·00	10	0·85
C	—	2	1·1	5	1·06
D	—	3	1·1	2	0·92
E	0·93	2	1·05	10	0·89
F	0·95	3	0·93	9	0·84
G	0·97	3	1·0	2	0·75

In conclusion I have to thank the Medical Research Council for the help I have received from them during the present investigation, also Prof. MacIlroy for giving facilities at the Royal Free Hospital for the investigation of suitable cases.

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LXXVI. THE RELATION BETWEEN THE PANCREAS AND THE CARBOHYDRATE METABOLISM OF MUSCLE.

I. THE EFFECT OF EXTRACTS FROM THE GLAND ON LACTIC ACID PRODUCTION *IN VITRO*.

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The first chemical research of importance which was inspired by Minkowski's classical discovery concerning the pancreatic function in metabolism was that of Otto Cohnheim [1904] who showed that the combined action of muscle tissue and a pancreatic extract on glucose leads to a fall in the reducing power. This result, which was confirmed and extended by the work of Hall [1907] was for some time interpreted as showing that a pancreatic factor determines the breakdown of sugar in the muscle. As is well known, however, Levene and Meyer [1911] showed that the reducing sugar disappears in these circumstances, as the result, not of a destruction, but of a synthetic process. They were able to demonstrate that a disaccharide is formed, and found that the original reducing power of the added sugar was restored after acid hydrolysis.

A somewhat different (though, almost certainly, related) effect of a pancreatic factor was later demonstrated by Winfield and Hopkins [1915] who showed that pancreatic preparations can inhibit the formation of lactic acid in chopped muscle. Owing to the circumstances of the time, this work could not then be continued. At Professor Hopkins' suggestion, we have now studied the phenomenon more closely.

The source of the pancreas as used by Winfield and Hopkins was found to be immaterial; the maximum effect was obtained by using a commercial preparation of pancreatin. The maximum inhibition of lactic acid formation that they report is one of 30 %, and the average is only 17 %, but it must be remembered that the only method of estimation then available was that devised by Fletcher and Hopkins. This requires relatively large quantities of muscle, and it is impossible during the removal and manipulation of so much mammalian muscle to prevent the development of considerable quantities of

lactic acid. There is no conclusive evidence given in their report that the inhibition of the lactic acid formation was not due to the simple digestion of the muscle by trypsin added with the pancreatic preparation, except that they state that the factor is heat-stable. An examination of their figures, however, shows that there was a considerable diminution of inhibitory power after boiling. In one case only a 2 % inhibition was found, and it has been shown by Mellanby and Woolley [1913], that in the presence of protecting proteins, trypsin can resist boiling for some time. It was to confirm the existence of this inhibitory factor, to show that it is not trypsin, and also to see if the inhibitor and insulin are one and the same substance, that this present work was undertaken.

Preparation of the Gland Extract.

It was considered advisable to use the same pancreatic preparation throughout the whole series of experiments. With this end in view the Hardy-Gardiner [1910] technique as modified by Young [1922] for obtaining a dry, unchanged, and soluble protein was applied to the pancreas. About 2 lbs. of pig's pancreas obtained from a commercial firm was freed from fat, minced, and placed in cold store at – 6° overnight. The next day it was placed in a stoppered jar and covered with three to four times its volume of 97 % alcohol previously cooled to the same temperature. It was allowed to stand for two days with occasional shaking and the alcohol then changed. This was repeated once more with 97 % alcohol; then twice with an equal volume of absolute alcohol; finally twice with absolute ether, each time the minced gland being filtered through a fluted paper and replaced in the jar. The gland was then transferred to a dish and dried in a desiccator, all these processes being carried out at the same temperature, viz. – 6°. Finally, still in the desiccator, it was brought up to the temperature of the laboratory, and then extracted for three days in a Soxhlet with absolute ether and dried in a vacuum desiccator. The dry residue was rubbed through a fine sieve to break up all lumps and remove connective tissue, etc.

The resulting white powder is hygroscopic, almost completely soluble in water or dilute saline giving a clear, faintly yellow, solution with a very high protein concentration, as shown by the heavy coagulum on boiling. It contains trypsin, amylase and lipase in high concentration and in a very active form. The presence in it of insulin has been demonstrated and also, as will be shown below, the factor responsible for the inhibition of lactic acid formation in muscle. Such a preparation has been kept in an ordinary stoppered bottle on the laboratory shelves for nine months with all its properties unimpaired, and there seems no reason why it should not keep indefinitely so long as moisture is excluded. This preparation is to be recommended as a method of making and storing trypsin and pancreatic amylase in a concentrated and stable form, and may prove useful in the case of other enzymes. It was hoped by using a perfectly fresh pancreas to obtain trypsin in the form of its

inactive precursor trypsinogen, but though a pancreas was obtained immediately on the death of the animal, and frozen at once, the resulting dry powder contained as much trypsin as the earlier specimen. In this preparation 150 g. of the fresh gland gave 25 g. of the dry extract before the sieving process, and 14 g. afterwards.

Experimental Details.

Throughout this work beef was used and it was always prepared in a standard way. An assistant was present at the slaughter house when the beast was killed, and immediately on the death of the animal, a piece of the neck weighing about 2 lbs. was cut out and placed in a pan, surrounded by a freezing mixture, and was then brought straight to the laboratory. It remained untouched until the temperature had fallen to about 5° or 6°. The fat was then removed and a piece of muscle, as intact as possible, dissected out, minced, and at once cooled in ice. Portions of 4 g. were weighed out on a tared watch glass and transferred quantitatively to small (150 cc.) conical flasks containing the buffer solution, pancreatic extract, etc. The flasks were well shaken, closed by cotton wool plugs, and then placed in the thermostat, or allowed to stand at room temperature according to the conditions of the experiment. The buffer solution used was acid potassium phosphate, adjusted to the required hydrogen ion concentration by the addition of 0·2 *N* NaOH. The concentration of phosphate used was double that recommended by Clark and Lubs, as this gives a solution approximately isotonic with muscle and the optimum conditions for lactic acid formation [Meyerhof, 1921]. The system was made anaerobic, and therefore any loss of lactic acid by oxidation prevented, by the addition of potassium cyanide, 1 cc. of an *N*/100 solution being added to both control and experimental flasks. According to Meyerhof [1921] this also acts as an antiseptic, and renders unnecessary the addition of other preservatives, such as toluene, which have an inhibitory effect on the lactic acid production.

At the end of the period of incubation, whether in the water-bath or at room temperature, the whole series of flasks were placed in ice to prevent any further change.

Estimation of the Lactic Acid.

The contents of each flask were filtered through muslin into a small beaker. The flasks were washed out with about 40 cc. of alcohol which was added to the filtrate. The muscle residue was then ground three times with sand and alcohol and the whole transferred to a second small beaker holding about 50 cc. Both beakers were allowed to stand overnight. The heavy protein precipitate was then filtered off, washed three times with alcohol, and the alcohol carefully evaporated on the water-bath, using a small beaker (A) and avoiding boiling. The alcohol from the muscle residue was evaporated with similar precautions in another small beaker (B), the residue being reground three times and the washings added to the filtrate. All alcoholic

extracts were made faintly acid with sulphuric acid before evaporation. When the residue in A was nearly, but not quite, dry it was once more extracted three times with alcohol, filtered, and the filtrate added to beaker B. In this way a residue was obtained almost free from protein, phosphates, etc. The remainder of the procedure was as described by Meyerhof [1920] with slight modifications. In all cases the final solutions were made up to 50 cc. and kept at − 2° until the actual distillation, for which 10 cc. were always used. The results are all expressed as percentages of lactic acid. In every case duplicate estimations were made[1].

Preliminary Experiments.

Exp. 2. A solution of the dried gland in water was made, filtered, and different quantities added to a series of flasks. 4 g. of minced beef were placed in each of seven flasks containing 10 cc. of phosphate solution at p_H 5·8. 0·1 g. glucose was added to each and also the pancreatic extract as indicated in the table. All specimens were placed at 37° for 30 minutes to allow for any possible time factor [Dakin and Dudley, 1913] and also to prevent destruction of insulin by a more alkaline reaction. At the end of that time 2·1 cc. of 0·2 *N* soda (sufficient to bring the p_H to 7·0) and 1 cc. of *N*/100 KCN were added to each flask and the series incubated at 37° for two hours. A determination of the initial lactic acid content of the beef was made on a separate sample.

Table I.

No.	Amount of dry gland equivalent to extract added	cc. of 0·0102 *N* iodine equivalent to 10 cc. of the final solution	% lactic acid	Effect
Exp. 2.				
1	Control (none)	10·95	·63	—
2	,,	10·20	·59	—
3	10 mg.	5·65	·33	59 % inhibition
4	,,	5·80	·34	57 % ,,
5	4 mg.	5·95	·34	57 % ,,
6	,,	6·30	·36	53 % ,,
7	10 mg. heated to 85° for 10 minutes	7·20	·41	47 % ,,
8	Preformed lactic acid in sample of beef	2·45	·14	Increase due to incubation in control = ·470 %
Exp. 3.				
1	Control	10·90	·62	—
2	,,	10·65	·61	—
3	50 mg.	5·30	·31	50 % inhibition
4	,,	5·30	·31	50 % ,,

Similar results were obtained in another experiment using a much stronger extract of the dry gland.

Exp. 3. All the details of the procedure the same as in Exp. 2.

There is clearly an inhibitory effect due to the extract, but it was shown

[1] In spite of repeated attempts we have had to abandon the shortened method of Meyerhof [1921] as too unreliable. No matter how highly purified a specimen of ammonium sulphate is used, the presence of so much of this salt in the final distillation mixture may cause a blank as high as 3 cc. of *N*/100 iodine. It has also been our experience that consistent distillation figures are difficult to obtain with this method, the ammonium sulphate not only giving rise to a blank but to a variable blank.

that this extract contained large quantities of trypsin, and therefore there is no evidence that the failure to develop as much lactic acid as in the controls was not due to digestion of the muscle by this enzyme. In order to show that the inhibition was not due to trypsin, it was necessary to prepare an extract containing the inhibitory factor, but known to be free from trypsin. The presence or absence of trypsin was demonstrated by the use of caseinogen solution as described in Cole's text-book.

The use of heat and alkali was avoided because their effect on the inhibitory factor was unknown, and it is difficult to be sure that the destruction of the enzyme is complete. It was not found possible to remove the trypsin by adsorption methods. Kaolin, alumina, charcoal and silicic acid were all tried without effect. Two methods were however found to be successful. In the first the trypsin was effectively neutralised by the addition of fresh serum. The serum was added to a watery extract, and the mixture tested for trypsin as before. The results of Exp. 6 are given in Table II.

Table II.

No.	Pancreatic extract	Serum cc.	Saline cc.	Trypsin	cc. 0·0105 *N* iodine equivalent to 10 cc. of final solution	% lactic acid	Effect
5	Control (none)	1	5	–	6·00	·35	—
7	,,	0	6	–	5·60	·33	—
1	Watery extract equivalent to 25 mg. dry gland	1	0	–	4·60	·27	26 % inhibition
3	,,	1 boiled	0	+	4·90	·29	21 % inhibition

In the second method the effect of extracting the dry gland preparation with alcohol of different strengths was tried. This was found to be the most successful and convenient method for removing the trypsin and was the one finally adopted for routine work. Full details of the preparation of such an extract are given below, but in Table III are given the results obtained by using extracts of the gland with alcoholic solutions of different strengths. All details of experimental procedure are as in Table I.

Table III.

No.	Nature of extract	Trypsin	cc. of 0·0104 *N* iodine equivalent to 10 cc. final solution	% lactic acid	Inhibition
Exp. 3, No. 4	80 % alcoholic equivalent to 50 mg. dry gland	–	8·90	·52	+
	Control		10·65	·64	
Exp. 3, No. 5	80 % alcoholic equivalent to 25 mg. dry gland	–	10·55	·63	–
	Control		10·65	·64	
Exp. 4, No. 1	70 % alcoholic equivalent to 60 mg. dry gland	–	4·10	·24	+
Exp. 4, No. 2	60 % alcoholic equivalent to 60 mg. dry gland	+?	4·10	·24	+
	Control		6·50	·38	
Exp. 6, No. 15 and 16	70 % alcoholic equivalent to 25 mg. dry gland	–	3·90	·23	+
	Control		6·00	·35	

A 50 % alcoholic extract was found to contain trypsin and was therefore not used. These preliminary results show that there is an inhibitory factor other than trypsin present in the pancreas. It can be extracted by alcohol solutions up to 80 %, but a 60 % alcohol also extracts traces of trypsin and an 80 % alcoholic extract was found to be not so rich in the factor as one made with 70 % alcohol. The last is at the same time free from trypsin. In all later experiments, therefore, 70 % alcohol was used to extract the inhibitory factor from the dried gland and unless otherwise stated it is understood that the extract was prepared in the following way, although the actual amount of dry gland used varied.

Five grams of the dried gland were ground with 150 cc. of 70 % alcohol and allowed to stand with frequent shaking for 24 hours. The mixture was then filtered and the residue washed with about 25 cc. of 70 % alcohol. The filtrate and washings were evaporated *in vacuo* at 37° until the volume was reduced as much as possible. It is not possible to continue till all the alcohol has distilled off because of the excessive frothing on which even caprylic alcohol is without effect. The volume can generally be reduced to between 50 and 60 cc. in this way. The milky residue was then poured into centrifuge tubes and centrifuged for about ten minutes. By this procedure a small quantity of fatty material can be removed. The remainder was got rid of by shaking three times with an equal volume of ether (the residue after evaporation of the ether was shown to be exceedingly rich in phosphorus) which also removed the remaining alcohol. The ether was removed by careful warming, taking care that the temperature never rose above 40°, and by blowing an air current across the surface. The resulting watery solution was made up to 50 cc. It is usually quite clear, or only faintly opalescent, and colourless. The experiments to be described show that it contains the inhibitory factor in high concentration. As a rule, the extract was made up fresh for each experiment, but one extract kept at – 6° for about six weeks still retained its inhibitory action almost unimpaired.

Experiments with Trypsin-free Extract.

1. *With increased Lactic Acid Production.*

Laquer [1914] showed that the so-called "acid maximum" of frog's muscle was merely the result of the conditions in the dead muscle and was really reached by self-hindrance in the reaction. By suspending the chopped muscle in an alkaline solution this "acid maximum" could be raised considerably. Embden and his collaborators [1914] showed that another limiting factor was the amount of the hexosephosphate precursor available and Meyerhof [1921] found that by suspending chopped muscle in a phosphate buffer solution at p_H 9·0, the quantity of lactic acid formed could be increased up to about 1 %. A series of experiments were therefore carried out under the conditions likely to yield high concentrations of lactic acid in order to

discover what degree of inhibition could be produced. The technique of the experiments was the same as for those already described, except that instead of being incubated at 37° for two hours, the flasks were allowed to stand for 24 hours at room temperature. Also in these and later experiments no glucose was added to the muscle mixture. The phosphate solutions were adjusted to p_H 7·0 and 9·0 respectively by the addition of 0·2 *N* soda. In the latter case the additional soda was not added until 30 minutes had elapsed from the time of adding the extract. Potassium cyanide was added as before. The protocol of one Exp. (7) is given in Table IV.

Table IV.

No.	Inhibitory factor	Saline cc.	Phosphate	cc. of 0·0106 *N* iodine equivalent to 10 cc. final solution	% lactic acid	Inhibition %
1 and 2	Control (none)	6	10 cc. at p_H 9·0	15·9	·95	—
3 and 4	„	6	„ p_H 7·0	16·1	·97	—
5 and 6	1 cc. 70 % extract = 50 mg. dry gland	5	„ p_H 9·0	10·8	·65	32
7 and 8	„	5	„ p_H 7·0	10·6	·64	33

These figures show that even in the optimum conditions for the formation of lactic acid, the pancreatic factor exercises an inhibitory power and later experiments (Tables VI to X) show an even greater inhibition.

2. *Experiments to show that the inhibitory effect is not due to insulin.*

It has now been proved that there is in the pancreas a factor capable of inhibiting the production of lactic acid in chopped muscle and that this factor is not trypsin. It now remains to show that the inhibition is not due to insulin, which is also present in the pancreas and is also associated with carbohydrate metabolism. Both aqueous and 70 % alcoholic extracts of the dried pancreas might be expected to contain insulin, and that such is the case is shown by the following figures. Rabbits were used as experimental animals, and the blood sugars estimated by a modification of the Folin-Wu colorimetric method.

Table V.

Date	Rabbit	Time	Blood sugar %	Remarks
20. vii. 23	I	10·12	·14	
		10·25	—	Injected with aqueous extract equivalent to 1 g. of the gland preparation
		11·18	·07	
		12·55	·11	
26. vii. 23	II	11·50	·10	
		12·00	—	Injected subcutaneously with a similar dose of aqueous extract made isotonic with sodium chloride
		12·55	·08	
		2·00	·065	
		3·55	·04	
		5·00	—	Convulsed
		5·10	·03	
		5·15	—	Recovered by subcutaneous injection of glucose
17. viii. 23	XI	10·35	·133	
		10·55	—	Injected with alcoholic extract ≡ 1 g. dry gland
		11·55	·067	
		2·10	·052	
		4·45	·073	

The 70 % alcoholic extract of the gland preparation has not yet been further fractionated, but it has been shown that insulin has no effect whatever on the lactic acid formation. The first experiments on this question were carried out by Miss D. M. Moyle working in this laboratory. Her results may be summarised as follows:

The experimental mixtures were left at p_H 7·1 for 3½ hours at 37°.

(*a*)	Control	13·05 cc. iodine	≡·62 %	lactic acid
	+3 cc. insulin	13·20 cc. „	≡·63 %	„
(*b*)	Control	9·35 cc. „	≡·44 %	„
	+2 cc. insulin	9·90 cc. „	≡·47 %	„

(Two samples of crude insulin were mixed and 3·8 mg. of the mixture dissolved in 5 cc. of saline.)

These results were repeated and confirmed by us, and the effect of insulin compared with that produced by the 70 % extract. The results are given in Table VI. Two samples of insulin were used, each of which was known to be active. One was the purest commercial sample obtainable, and one therefore in which any possibility of contamination with the inhibitory factor was reduced to a minimum. The other was a specimen of crude insulin (for which we are indebted to Messrs Winter and Smith) which had undergone no purification. Two samples also of the 70 % extract were used. One was freshly prepared and the other had been kept in cold store at − 6° for nearly two months. It seemed of interest to test this old extract (the same as that used for Exp. 7) for deterioration of the factor, because it was found that it had no longer any insulin-like effect on the blood sugar when injected into a rabbit. Exp. 7 had shown that a p_H of 7·0 gave as high values for the lactic acid content of muscle in the controls as p_H 9·0, so it was decided to work at the neutral point, since insulin is readily destroyed on the alkaline side. The insulin was added while the contents of the flasks were still acid at p_H 5·8, and soda was added after half-an-hour to make the p_H 7·0. Otherwise, all experimental details were as in Exp. 7. (*Note.* The lactic acid in this series was estimated by the shortened method of Meyerhof, referred to above, and the figures given are in each case the mean of two samples. The figures in brackets are the extreme values obtained in the distillations.)

Table VI. *Exp.* 10.

No.	Factor added	cc. of 0·0102 *N* iodine equivalent to 10 cc. of final solution	% lactic acid	Inhibition %
1 (*a*)	Control	14·60	·84	—
(*b*)	1 cc. saline	(14·40, 15·15)		
2 (*a*)	1 clinical unit pure commercial insulin	14·10	·81	—
(*b*)		(14·20, 13·65)		
3 (*a*)	5 mg. crude insulin	14·90	·86	—
(*b*)		(14·90)		
4 (*a*)	1 cc. old 70 % extract equivalent to	6·90	·40	52
(*b*)	100 mg. dry gland	(7·25, 6·35)		
5 (*a*)	1 cc. new 70 % extract equivalent to	5·30	·31	63
(*b*)	125 mg. dry gland	(5·70, 4·90)		

It is clear that the factor in the pancreas responsible for the inhibition of lactic acid formation in chopped muscle is not insulin, and it is also evident that even in crude samples of insulin, the inhibitory factor has been removed by the higher concentration of alcohol used in its preparation.

3. *Antiglyoxalase.*

There is also present in the pancreas another factor of which no mention has yet been made, and which is probably concerned with carbohydrate metabolism and with lactic acid production, and that is the antiglyoxalase discovered by Dakin and Dudley [1913]. These authors showed that there is present in practically all tissues, except the pancreas, an enzyme, glyoxalase, which is able to convert α-ketonic aldehydes into the corresponding optically active hydroxy-acids.

$$R . CO . CHO \rightarrow R . CHOH . COOH.$$

Thus lactic acid would be produced by glyoxalase from methylglyoxal.

$$CH_3 . CO . CHO + H_2O \rightarrow CH_3 . CHOH . COOH.$$

They found that the pancreas and commercial pancreatin, on the contrary, contain an antagonistic substance, called by them antiglyoxalase, which inhibits the action of glyoxalase. If a ketonic aldehyde is added to a tissue preparation, together with a pancreatic extract, there is no formation of the corresponding acid. Thus, in so far as it inhibits the production of lactic acid, antiglyoxalase would appear to be analogous, if not identical, with the inhibitory factor described above. Although possible however, it has not yet been definitely established that methylglyoxal is one of the intermediary products in the breakdown of hexosephosphate to lactic acid in the muscle. To decide, if possible, whether methylglyoxal occurred as an intermediary in lactic acid production was (we are informed) the original intention of Winfield and Hopkins' experiments. Antiglyoxalase is described as a thermolabile body, being destroyed by heating for ten minutes at 85°. It is slowly destroyed by weak acids, but is much more resistant to alkali. Even after 24 hours' digestion at 37° with 1 % sodium carbonate, its inhibitory action on glyoxalase is unimpaired (Dakin and Dudley). In order to decide conclusively whether the inhibitory factor we have described is, or is not, identical with antiglyoxalase, it will be necessary to test its action on phenylglyoxal added to a tissue mixture. It is hoped to be able to do this shortly, but meanwhile its resistance to alkali was compared with that attributed to antiglyoxalase.

Extracts of the dried gland, both with water and 70 % alcohol, were made up to 1 % with solid anhydrous sodium carbonate, a drop or two of toluene added, and the mixtures placed in the incubator overnight at 37°—usually for about 20 hours. They were then neutralised with hydrochloric acid and added to minced beef suspended in phosphate in the usual way. The results are summarised in Table VII.

Table VII.

No.	Extract	Conditions	cc. iodine equivalent to 10 cc. final solution	% lactic acid	Inhibition
Exp. V 1	Control	Heated at 37° for 2 hours at p_H 7·0	7·10	·42	—
2	50 mg. commercial pancreatin	„	4·90	·29	Marked
3	50 mg. pancreatin digested with 1 % Na_2CO_3	„	6·30	·37	Slight
Exp. VI	Control	„	5·60	·33	—
9	1 cc. 70 % extract = 25mg. dry gland digested with 1 % Na_2CO_3	„	6·00	·35	None
14	Aqueous extract = 25 mg. dry gland digested with 1 % Na_2CO_3	„	5·60	·33	None
Exp. VII	Control	Room temp. for 24 hours at p_H 9·0	15·90	·95	—
9 and 10	1 cc. 70 % alcoholic extract = 30 mg. dry gland digested with 1 % Na_2CO_3	„	16·10	·97	None
Exp. XI 1	Control	Room temp. for 22 hours at p_H 7·0	12·50	·71	—
2	1 cc. 70 % extract = 100 mg. dry gland untreated	„	5·30	·28	Marked
5	1 cc. 70 % extract digested with 1 % Na_2CO_3	„	10·85	·63	Slight

Though not entirely conclusive these results certainly suggest that in its inability to resist the action of alkali the pancreatic factor responsible for the inhibition of lactic acid formation in chopped muscle differs from antiglyoxalase.

4. *Stability towards heat.*

Too few figures have been obtained yet to say definitely how resistant towards heat the inhibitory factor is. Dakin and Dudley found that antiglyoxalase was completely destroyed by exposure to a temperatue of 85° for ten minutes. Winfield and Hopkins state that the factor is thermostable and will withstand boiling. As already noted, in their preparations, the enzymes may have been protected by the proteins present, and this is also the case in the first experiment quoted below. An aqueous extract of the gland preparation gives a heavy coagulum on being heated, but this possible source of error is absent in the second experiment. The alcoholic extract remains unclouded at the end of ten minutes at 85°.

In each case there has been a considerable destruction of the factor, but this seems not to be complete. Further work must be done to find to what extent it is thermostable, but in respect to heat also it would appear to be more resistant than antiglyoxalase.

Table VIII.

No.	Extract	Conditions	cc. iodine equivalent to 10 cc. final solution	% lactic acid	Inhibition %
Exp. III	Control	Incubated for 2 hrs. at 37° at p_H 7·0	10·60	·61	—
3 and 4	Aqueous extract equivalent to 10 mg. dry gland	„	5·70	·33	46
7	Same heated to 85° for 10 mins.	„	7·20	·42	31
Exp. XI	Control	Room temp. for 22 hours at p_H 7·0	12·50	·72	—
2	1 cc. 70 % alcoholic extract = 100 mg. dry gland	„	5·30	·30	58
9	Same heated at 85° for 10 mins.	„	11·50	·66	8

Another interesting feature of the action of the inhibitory factor is illustrated in the results given in Table IX. Estimations were made on samples of beef to which the extract was added after the most part of the lactic acid had been formed. The object of such experiments was to ascertain whether the diminished yield of lactic acid produced by the factor was due to a hindrance in the formation of the lactic acid or to its removal, either by oxidation or by synthesis to a precursor, after its formation. It was found that when once the lactic acid had been liberated, under the conditions of our experiments the factor had no effect on it. This would suggest that its action is truly one of inhibition and its effect is not to catalyse the removal of the acid.

Table IX.

No.	Extract	Conditions (all at p_H 7·0)	cc. iodine equivalent to 10 cc. final solution	% lactic acid
Exp. X 7	Control	At 37° for 2 hrs. and then 22 hrs. at room temperature	15·30	·88
6	1 cc. 70 % extract equivalent to 125 mg. dry gland	At 37° for 2 hrs. then extract added and left for 22 hrs. at room temperature	12·80	·74
Exp. XI 6	Control	At 37° for 2 hrs.	12·15	·70
7	Control	At 37° for 2 hrs. then 1 cc. saline added and left at room temperature for 20 hrs.	14·40	·83
8	1 cc. 70 % extract equivalent to 100 mg. dry gland	At 37° for 2 hrs. then extract added and left at room temperature for 20 hrs.	13·10	·75

In Table X are summarised the results obtained in one experiment showing the effect of concentration, alkali, and heat on the same extract. All samples were kept for 22 hours at room temperature which varied from about 10° to 13° during the experiment, and all were at p_H 7·0. The details of technique were exactly as already described[1].

[1] In all tables, except no. 1, the percentage inhibitions given are really less than the actual inhibitions, because there is always some 'preformed' lactic acid, the amount of which is the true base line for the changes. This follows from the results given in Table IX.

Table X. *Exp.* 11.

No.	Extract	cc. 0·0102 *N* iodine equivalent to 10 cc. final solution	% lactic acid	Inhibition %
1	Control	12·50	·72	—
2	1 cc. 70 % alcoholic extract equivalent to 100 mg. dry gland	5·30	·30	58
3	0·5 cc. extract equivalent to 50 mg. dry gland	5·30	·30	58
4	0·25 cc. extract equivalent to 25 mg. dry gland	6·10	·35	52
5	1 cc. extract digested with 1 % Na_2CO_3	10·85	·62	14
9	1 cc. extract heated to 85° for 10 mins.	11·50	·66	8

There are one or two interesting properties of the factor which might be noted. It is never able to prevent entirely the formation of lactic acid. In every case, however much of the preparation was added, there was some formation of the acid. For example, assuming the initial value of the "preformed" lactic acid as 0·14 % (Table I) the lowest value we have obtained after addition of the factor and subsequent incubation was 0·23 % (Table III). Also an increase of 100 % in the amount of factor added, above a certain minimum, had no further effect in inhibiting the lactic acid production. Then again, many experiments show a suggestive proportionality between the total lactic acid formed and the degree of inhibition. In our opinion these results lend some support to the view of Meyerhof [1921] that there is more than one source contributing to the total lactic acid production in muscle. In may well be that the inhibitory factor is only capable of exerting its action on one of these sources, though with the data available, this can be nothing more than a suggestion.

Discussion of Results.

The experiments described show conclusively that there is present in the pancreas a factor which can control the production of lactic acid in muscle by exercising a marked inhibitory action. All the data available at present refer, it should be understood, to its action on chopped muscle *in vitro*, and to that alone. The factor is not insulin. It would seem moreover to differ from the pancreatic substance which was originally described by Otto Cohnheim and was later shown by Levene and Meyer to induce, when mixed with muscle, disaccharide formation from glucose. The active substance studied by us is, to judge at least from the published statements, less soluble in strong alcohol and less thermostable than Cohnheim's substance. It is possible however that these differences may be due to variations in the method of extraction. There is some evidence, as we have pointed out, to suggest that our inhibitory factor is not identical with the antiglyoxalase of Dakin and Dudley.

There is as yet no evidence as to what the exact physiological functions of this new factor may be but certain speculations are perhaps permissible and are certainly interesting. An attractive suggestion is that it forms part

of a delicate regulating mechanism in which insulin is also included. Since it inhibits the formation of lactic acid there is at least a possibility that it may exercise a stabilising influence on the glycogen stores of the body; in the muscles if not in the liver. If this be the case then insulin and the inhibitory factor may be, in this particular connection, mutually antagonistic, for as Dudley and Marrian [1923] showed, when insulin is injected into a normal animal the glycogen almost disappears from the liver and muscles. It has been shown by Robison and Kay [1924] that in the living animal, insulin has the power of increasing the lactic acid precursor—lactacidogen—which in its turn would normally result in an increased lactic acid production. Is this effect balanced and controlled in the normal animal by the intervention of the inhibitory factor? In the experiments where insulin was added to the muscle *in vitro* no increased lactic acid formation over the control was observed. Under these conditions, however, there would be an insufficient supply of the precursor, for though phosphate was supplied, carbohydrate was not and the development of lactic acid would cease when the precursor was exhausted, whether by the normal enzymes of the muscle or jointly by these and insulin. The effect of insulin upon the velocity of lactic acid production was not studied.

It has always been an unexplained circumstance that the muscular metabolism of the diabetic shows no obvious abnormality. There have been attempts to estimate the lactic acid in diabetic muscle and though the results are somewhat contradictory the general view is that it differs little, if at all, from the normal. The energy supply, even in the diabetic, is immediately derived from the breakdown of carbohydrate. Is this because the breakdown of glycogen through hexosephosphate and lactic acid in the muscle follows a different course from the general carbohydrate metabolism of the body? Is the latter regulated by insulin which controls the concentration of glucose in most tissues, while the specialised events in the muscles, where the lactic acid is not only part of the "fuel" but also of the "machinery" are regulated by this new factor?

These suggestions as to the physiological functions of the inhibitory factor are of course purely tentative and highly speculative, but we consider that they warrant our continued research into its effect on the intact animal by administration alone and with insulin and it is hoped the results will form the subject of a later communication.

Summary.

The presence in the pancreas of a factor which is capable of causing marked inhibition in the lactic acid production of chopped muscle, is shown.

This inhibitory factor is neither trypsin nor insulin and probably not antiglyoxalase.

A method is described for making a stable preparation of the pancreas with all its enzymes intact and details are given for making an extract of this

preparation with 70 % alcohol which contains the inhibitory factor in high concentration.

We wish to thank Professor Hopkins for suggesting this research and for his continuous interest and help. We are also indebted to Miss D. M. Moyle for permission to publish her figures.

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LXXVII. THE CHEMISTRY OF AMINO-ACID DEAMINATION.

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The final phase of amino-acid metabolism, involving the removal of nitrogen from the amino-group and the production of urea, is generally regarded as involving two distinct processes: (1) deamination, with the liberation of ammonia, (2) dehydration of ammonium carbonate, with formation of urea.

The change being represented as:

$$R.CH(NH_2).COOH \longrightarrow NH_3 \longrightarrow (NH_4)_2CO_3 \longrightarrow CON_2H_4$$

Now, while there is no doubt as to the general character of the transformation, the chemical changes involved in each intermediate process are somewhat obscure. The present paper is concerned chiefly with the first process, but it will be seen that the conclusions arrived at afford an indication as to the mechanism of the formation of urea from ammonium salts.

Deamination by α-oxidation.

Neubauer [1908] has indicated the three most direct methods by which an amino-acid may undergo deamination. These are: (1) oxidation, with formation of the corresponding α-ketonic acid; (2) reduction, with formation of the corresponding saturated acid; (3) hydrolysis, with formation of an α-hydroxy-acid.

Chemical and physiological evidence, as summarised by Dakin [1922], supports strongly the belief that in the animal organism deamination is chiefly, if not entirely, brought about by oxidation; and such observations as those of Fiske and Karsner [1914], and Jansen [1915] that aeration of the perfusion fluids is necessary for the removal of ammonia and amino-acids is of interest in this connection. Quite apart from the subject of protein metabolism, there is ample proof that when certain amino-acids are oxidised in neutral or alkaline solution the amino-groups ultimately appear as ammonia.

If the group $-NH_2$ be assumed to be converted directly into NH_3, the general difficulty arises as to the liberation of ammonia by oxidation in alkaline solution. Ammonium salts are very resistant towards oxidation when in strongly acid solutions, as, for example, in the familiar Kjeldahl process.

In alkaline solution, and in presence of carbon compounds, ammonia readily undergoes oxidation, as emphasised by the extensive investigations of Fosse [1919, 1920, 1921, 1922]. This difficulty of accounting for the production of ammonia by oxidation has already been raised by Werner [1923] in connection with the origin of urea in the animal body, and has been anticipated by Lippich [1908] and by Dakin [1909], both of whom have considered the possibility of deamination without liberation of ammonia, but with the formation of intermediate uramido-acids.

As Fiske and Sumner [1914] have observed: "It cannot be stated with certainty that ammonia is an obligatory intermediate product in the deamination in mammals."

A second and more special difficulty arises in connection with the theory of α-deamination. It is necessary to account for the removal of the amino-group by oxidation without breaking the carbon chain. Knoop [1910] has suggested that this is accomplished through the formation of a hypothetical intermediate body, a hydrated amino-acid, but this suggestion does not meet the general difficulty of accounting for the direct formation of ammonia by oxidation.

The formation of cyanic acid by oxidation.

Cyanic acid is so intimately associated with urea and its various syntheses outside the organism that Werner [1923] has been led to conclude that the mere occurrence of urea in the animal body implies the formation of cyanic acid at some stage in protein metabolism, which is supported by the detection of cyanic acid in dog's blood by Nicloux and Welter [1922].

Cyanic acid has been identified by Fosse [1921] during the oxidation of a great variety of organic nitrogen compounds, and appears to be of much more general occurrence than is at present realised. The discovery by Walters and Wise [1917] of cyanuric acid, the polymer of cyanic acid, in soil may be noted in this connection.

With the object of investigating the possible relationship between deamination by oxidation and cyanic acid formation, the behaviour of four aliphatic amino-acids on oxidation in alkaline solution was studied. The results obtained support the view that deamination may be brought about by the intermediate formation of an unstable cyanate.

Conditions of deamination.

The conditions which may be expected to determine the course of deamination both inside and apart from the organism are as follows:

1. *The nature and concentration of the amino-acid.*

2. *The reaction of the solution.* In acid solutions cyanic acid is very readily hydrolysed. For purposes of investigation many of the solutions were kept in the region of p_H 8 to approximate to conditions of the animal body and to stabilise any cyanic acid formed by fixing it as a cyanate.

3. *The oxidation mechanism.* It is here that the reproduction of biochemical oxidations presents the greatest difficulty. The important part played by the tissues is very hard to define and to imitate. Dakin [1922] has summarised the reasons for considering that oxidation by hydrogen peroxide most closely resembles the oxidations effected by the organism.

Accordingly, hydrogen peroxide was first employed in the present investigation. In strengths from 3 to 20 %, in varying proportions, with and without a catalyst, the action was found to be feeble at room-temperature and at 45°. The results obtained by Dakin [1906, 1] are probably due to his procedure of distillation in presence of undecomposed peroxide, a method which is not suitable for a solution which is later to be tested for cyanate. For these reasons, potassium permanganate was employed in the later experiments.

4. *The presence of glucose.* It is worthy of note that in the organism deamination occurs in presence of other substances which may affect the course of the reaction. Glucose, the normal constituent of blood, appears to be the most important of these possible accessories. By the addition of an equimolecular portion of glucose to glycine undergoing oxidative deamination the production of cyanate was increased ten times and the liberation of free ammonia was reduced from 48 % of the theoretical to 13 %.

Experimental.

Glycine (Kahlbaum), alanine (Kahlbaum), leucine, and sarcosine (Merck), were oxidised at room-temperature, 30° and 45°, in *M*/10 and *M*/50 concentrations, by means of neutral hydrogen peroxide, 3 % and 20 %, with a trace of ferrous sulphate, and by potassium permanganate, in *N* alkali or in *N*/15 disodium phosphate. The periods of oxidation were 24 hours, and for leucine 48 hours.

The excess of oxidising agent was then removed, and the extent of deamination determined by aspiration of the solution. The solution was then neutralised, and divided into equal portions. In one the urea was determined by evaporating to dryness, extracting with alcohol, and applying the xanthydrol reagent to the concentrated alcoholic extract according to the method of Fosse [1907].

The cyanic acid in the second portion was determined by incubation overnight with 1 g. of ammonium chloride, which interacted with the cyanate present to form urea; this was found to be more satisfactory than the method first adopted of evaporating the second portion of the extract together with the ammonium chloride to dryness on the water-bath, which led to loss of some of the cyanate by hydrolysis.

The urea formed from the cyanate was determined quantitatively by the xanthydrol method; the difference between the two urea values gave the amount of cyanate present in the solution.

The identity of the precipitate of dixanthylurea was established by microscopic appearance and melting-point in the vapour of diphenyl ether.

Control experiments showed that the amino-acids produced no urea or cyanate under the above conditions in the absence of oxidising agents.

It is necessary to free the solution completely from any unchanged peroxide or permanganate before applying the xanthydrol test, otherwise a precipitate of xanthone is formed which obscures the reaction.

Table I. *Glycine oxidation.* 24 *hours.*

	Weight of glycine g.	Oxidiser	Temperature	Ammonia	Cyanic acid	Urea
				(*percentage of theoretical*)		
1	0·75	H_2O_2 20 cc. = ·32 g. O	Air	1·6	Present	0
2	,,	H_2O_2 40 cc. = ·64 g. O	45°	4·5	,,	Present
3	,,	$KMnO_4$ 1 g., 0·1 *N* NaOH 20 cc.	Air	3·4	,,	,,
4	,,	$KMnO_4$ 1 g., *N* NaOH 20 cc.	,,	3·4	0·13	,,
5	,,	$KMnO_4$ 1·5 g., *N* NaOH 20 cc.	,,	—	0·95	,,
6	,,	$KMnO_4$ 1·5 g., *N* NaOH 20 cc.	45°	48·2	1·4	,,
7	0·15	$KMnO_4$ 0·36 g., 0·1 *N* KOH 20 cc.	,,	14·0	2·6	—
8	0·15 + 0·036 glucose.	$KMnO_4$ 0·36 g., 0·1 *N* KOH 20 cc.	,,	6·5	6·6	Present
9	,,	$KMnO_4$ 0·6 g., 0·2 *N* KOH 20 cc.	,,	13·0	10·5	,,

THE SIGNIFICANCE OF CYANIC ACID IN DEAMINATION.

The cyanic acid identified in the experiments here recorded may arise directly by a process of oxidative deamination, or as a secondary oxidation (in presence of a hydrocarbon residue) of the ammonia liberated by deamination. That this latter possibility must not be ignored is shown by the observations of Fosse [1919] who obtained a 70 % yield of urea by the oxidation of small quantities of glucose in presence of excess of ammonia, and who also detected cyanic acid as the intermediate product. That a secondary reaction completely accounts for the cyanate produced when glycine is oxidised does not appear likely, since cyanate has been detected during the mild oxidation of glycine when the ammonia liberated was only $1\frac{1}{2}$ % of the theoretical value for complete deamination.

Furthermore, in a solution of glycine which was slowly oxidised at 45° for a period of 48 hours, the resulting rise in ammonia was not accompanied by a corresponding rise in cyanate, which suggests that the ammonia is produced by the continuous hydrolysis of the cyanate at a rate depending on the conditions of the experiment.

A formation of cyanate from ammonia by oxidation in presence of a hydrocarbon residue is not necessarily impossible in the present experiments, but by itself it is inadequate to explain the mechanism of deamination, although it may account for one of the methods whereby ammonia is removed from the circulating blood.

To explain a formation of cyanic acid by oxidative deamination without rupture of the carbon chain, two possibilities must be considered, namely: oxidation of the amino-acid when in the cyclic form, or oxidation of the amino-acid in presence of another carbon compound.

In neutral solution, amino-acids may assume a closed-chain structure:

$$\begin{array}{l} \mathrm{R.CH{-}NH_2} \\ \quad\vert \\ \mathrm{COOH} \end{array} \rightleftharpoons \begin{array}{l} \mathrm{R.CH{-}NH_2} \\ \quad\vert\quad\vert \\ \mathrm{CO{-}O} \end{array}$$

Such a configuration might conceivably undergo direct oxidation to form cyanic acid and the aldehyde corresponding to the amino-acid:

$$\begin{array}{l} \mathrm{R.CH{-}NH_2} \\ \quad\vert\quad\vert \\ \mathrm{CO{-}O} \end{array} \begin{array}{l} \\ \longrightarrow \\ +\mathrm{O} \end{array} \mathrm{R.CHO + HNCO + H_2O}$$

This was investigated experimentally by oxidising glycine in almost neutral solution and in definitely alkaline solution, under which latter condition the amino-acid would exist entirely in the open-chain or salt form:

$$\begin{array}{l} R{-}CH{-}NH_2 \\ \quad\quad | \\ \quad\quad COONa \end{array}$$

The yield of cyanate was greater in this condition, which indicates that the cyclic configuration is not essential for the production of cyanic acid.

To determine the influence of the presence of other carbon compounds, glycine was oxidised together with glucose, and also with formaldehyde in equimolecular proportions. In each case the yield of cyanic acid was greatly increased, rising from 1·4 % of the theoretical to 10·5 %.

This can be most readily explained by a union between the aldehyde and the amino-group previous to oxidation:

$$\begin{array}{l} R.CH.NH_2 \\ \quad\quad | \\ \quad\quad COOH \end{array} + HCHO \longrightarrow \begin{array}{l} R.CH.N:CH_2 \\ \quad\quad | \\ \quad\quad COOH \end{array} + 2O \longrightarrow \begin{array}{l} R.CO \\ \quad\quad | \\ \quad\quad COOH \end{array} + HNCO + H_2O$$

The oxidation of such a methylene-amino-acid would account for the production of a ketonic acid by oxidative deamination. Further support for this view was obtained by the oxidation of sarcosine, which gave a greater yield of cyanic acid than did glycine under similar conditions, showing that the oxidation had attacked the linkage between the nitrogen and the asymmetric carbon of the substituted amino-acid. This is in agreement with Dakin's observation [1906, 2] that methylamine is formed during the oxidation of sarcosine.

Table II. *Sarcosine oxidation.* 24 *hours.*

	Weight of sarcosine g	Oxidiser	Temperature	Ammonia	Cyanic acid	Urea
				(*percentage of theoretical*)		
1	0·178	$KMnO_4$ 0·6 g., 0·2 *N* KOH 20 cc.	30°	21·5	3·0	—
2	0·178 + ·036 (glucose)	„ „	„	8·0	2·5	0

Table III. *Alanine oxidation.* 24 *hours.*

	Weight of alanine g.	Oxidiser	Temperature	Ammonia	Cyanic acid	Urea
				(*percentage of theoretical*)		
1	0·89	H_2O_2 20 cc. = 0·16 g. O	Air	1·5	Present	Present
2	0·178	$KMnO_4$ 0·75 g., 0·1 *N* KOH 20 cc.	30°	19·0	0	0
3	0·178 + 0·036 (glucose)	$KMnO_4$ 0·75 g., 0·1 *N* KOH 20 cc.	„	27·5	0	0
4	0·178 + 0·16 (CH_2O)	$KMnO_4$ 1·2 g., 0·1 *N* KOH 20 cc.	„	—	1·0	0

Table IV. *Leucine oxidation.* 49 *hours. Temperature* 30°.

	Weight of leucine g.	Oxidiser	Ammonia	Cyanic acid	Urea
			(*percentage of theoretical*)		
1	0·131	$KMnO_4$ 0·6 g., 0·2 *N* KOH 20 cc.	98	0	0
2	0·131 + 0·036 (glucose)	„ „	28	0	0

Oxidation of alanine and leucine.

When oxidised with hydrogen peroxide under the conditions described, alanine is only slightly attacked at air temperature, a yield of ammonia corresponding to 1·5 % of the theoretical being obtained after 24 hours. Cyanic acid and urea were both detected in the solution. On oxidising alanine with alkaline permanganate at 45°, the yield of ammonia was increased to about 20 %, as compared with 48 % in the case of glycine under the same conditions. When the alanine was oxidised at the higher temperature, neither cyanic acid nor urea was detected in the solutions, which may be due to alanine undergoing a form of deamination different from that of glycine, although addition of formaldehyde or formic acid to the alanine before oxidation led to a production of cyanic acid.

Cyanic acid has not yet been detected during the oxidative deamination of leucine.

Whether these results may be taken to indicate that, in general, glycine undergoes a form of deamination distinct from that of alanine or leucine, or whether they are due to the experimental difficulties of correlating the conditions of effective oxidation with those favourable for the detection of the unstable cyanic acid can best be determined by perfusion experiments. For the present, all one can conclude is that the oxidative deamination of glycine and sarcosine involves the intermediate formation of cyanic acid.

The significance of cyanic acid in the organism.

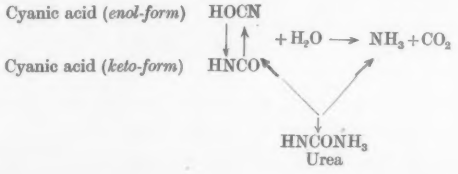
The formation of cyanic acid as an intermediate stage in deamination not only offers an explanation of the mechanism of deamination but has several very important possibilities in connection with the origin of urea.

Cyanic acid is stable towards oxidising agents, and once formed is unlikely to undergo further oxidative changes. It is, however, very reactive in two ways of great biological significance. It may undergo hydrolysis to produce ammonia and carbon dioxide, or it may unite with ammonia to form urea.

In presence of acids, cyanic acid is completely hydrolysed to ammonia:

$$HOCN + H_2O + HX \longrightarrow NH_3HX + CO_2$$

In aqueous solution alone, cyanic acid is in part hydrolysed to ammonia and carbon dioxide, and in part converted into urea by union with ammonia [Werner and Fearon, 1920]:



In presence of excess of ammonia, cyanic acid is ultimately completely converted into urea:

$$\begin{array}{lll} HOCN + NH_3 \rightleftarrows NH_4CNO & & \text{Ammonium cyanate.} \\ \downarrow \uparrow & & \\ HNCO + NH_3 \longrightarrow HNCONH_2 & & \text{Urea.} \end{array}$$

This unique property of cyanic acid, producing either a base or a neutral compound according to conditions of hydrolysis, suggests at once a more precise explanation of one of the factors for the maintenance of the neutrality of the organism. Thus may be explained the low concentration of ammonia in normal blood and the production of ammonia in conditions of acidosis, and also the formation of urea when a surviving liver is perfused with solutions of ammonium salts, as in the well-known experiments of Schröder.

Conclusions.

1. Cyanic acid is formed during the oxidative deamination of glycine, sarcosine, and alanine.

2. The α-deamination of glycine involves the intermediate formation of cyanic acid.

3. Cyanic acid must be considered as an important factor in the production of urea and in the maintenance of neutrality of the organism.

We wish to acknowledge our indebtedness to Prof. E. A. Werner for the interest he has taken in this research.

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LXXVIII. ON THE TYPE OF SUGAR EXCRETED IN THE URINE OF NORMAL PERSONS.

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For some considerable time, investigators have regarded the possibility of glucose passing through some intermediate form prior to utilisation in the body as very probable.

Hewitt and Pryde [1920] carried out experiments from which it appeared that glucose was to some extent converted into the γ-form by the intestinal mucous membrane.

Winter and Smith [1923] claimed to have shown that the form in which glucose circulates in the blood is that of γ-glucose, and they suggest that the transition into glucose is essential for utilisation in the organism. In diabetic patients, who are more or less unable to utilise glucose, these authors found that the sugar present in the blood was the ordinary α-β-glucose.

The fact that administration of glucose by mouth increases the blood sugar concentration in the normal individual, while laevulose does not do so, but appears to be immediately removed from the blood, might be explained on the supposition that laevulose is more adapted for assimilation than glucose, and that, prior to utilisation, glucose and certain other sugars must pass into some form more nearly allied to laevulose. According to Bornstein and Holm [1922] laevulose produces a rise in the respiratory quotient immediately after ingestion, while glucose does not do so for 40 minutes or more. From this observation it would seem that laevulose is both more easily stored, and more easily oxidised, than glucose.

If the sugar normally circulating in the blood be γ-glucose, its absence in diabetes may be due to the lack of an enzyme for the conversion of the ordinary α-β-variety into this form as Winter and Smith have postulated. To come to a definite decision on this point is a matter of both scientific and clinical interest, but the problem is a very complex one, and many other explanations of Winter and Smith's experimental results have been advanced. The extremely reactive nature of γ-glucose must render its isolation from animal tissues in the unchanged form a matter of almost insuperable difficulty, and, as pointed out by Hewitt [1923], it is not easy to see how the optical activities of the protein-free filtrates obtained from the blood by Winter and Smith throw any light on the nature of the original blood sugar. On the other hand, it is now well known that the blood contains unstable reducing

complexes other than glucose, such as phosphoric acid esters, which tend to complicate the experimental results.

It would, therefore, seem that examination of the sugar present in the urine might yield more conclusive evidence than could be obtained from the blood, and on this assumption the experiments quoted below were carried out. If γ-glucose is the sugar of normal blood, then γ-glucose should be the sugar of normal urine, unless the kidney changes it during secretion. The amount of sugar present in normal urine, however, is too small for satisfactory investigation, and the problem presenting itself was how best to increase the urinary sugar in a normal subject. Since the healthy kidney does not allow sugar to escape into the urine except in very minute quantities, it was obvious that some means by which the kidneys of the normal individual would cause the blood sugar to appear in concentration in the urine would have to be employed in order to throw light on the nature of the blood sugar. As is well known, phloridzin produces a marked glycosuria in normal subjects, and it was resolved to take advantage of this fact and ascertain the nature of the sugar produced after the administration of this drug. Phloridzin should not cause any change in the nature of the blood sugar, since its mode of action in increasing the output of blood sugar in the urine apparently depends on the fact that it lowers the threshold value of the kidney.

Some hours previous to the experiment a good carbohydrate meal was taken. An injection of 0·005 g. of phloridzin dissolved in sterile water was then given subcutaneously. To eliminate any tendency for the normally acid urine to act on the sugar present, small doses of sodium bicarbonate, just sufficient to produce neutral or very faintly alkaline urines, were given in some cases. Shortly after the phloridzin injection, the bladder was emptied, and as soon as possible afterwards a specimen was obtained for analysis. Care was taken to prevent the urine remaining in the bladder longer than was necessary. These experiments were carried out on students and convalescent patients. The specimens of urine examined contained from 1·5 to 4 % sugar.

These urines were tested for the presence of γ-glucose which, along with other properties, has the power to decolorise potassium permanganate, and to reduce Fehling's solution in the cold.

In no instance did a specimen of urine obtained after phloridzin injection reduce Fehling's solution in the cold, nor did it ever show a greater reducing action on potassium permanganate than was exerted by any average normal urine. The sugar present possessed none of the labile properties usually assigned to γ-glucose.

Further investigations were carried out by comparing the copper reducing power with the polarimetric reading. Polarimetric examination was always conducted as soon as possible after the urine was passed, so as to avoid or minimise any possible change. The volumetric estimation of sugar was done by Bertrand's method.

Generally speaking, the percentage of sugar, as estimated from polarimetric

observations, was somewhat less than that obtained by Bertrand's method, but since the urine contains several reducing bodies besides sugar, this is only what might be expected. The difference, on the whole, was very slight. Examination of the urine at varying intervals later did not show any appreciable change in the polarimetric readings. The urine obtained from two diabetic patients who had marked glycosuria, but were practically free from ketosis, gave exactly similar results to those obtained with urines passed after phloridzin.

From these observations it is certain that the sugar present in the urine of normal individuals after phloridzin is not γ-glucose; indeed, it appears to be ordinary α-β-glucose. No difference could be ascertained between the sugar of diabetic urine and that passed as the result of phloridzin injection in the healthy subject.

Protocols of some of the experiments are annexed.

Results of experiments on sugar passed in normal and diabetic individuals.

(Numbers 1 to 8 inclusive in urine from normal subjects after phloridzin; numbers 9 and 10 in diabetic urines.)

No.	Result of Fehling's test in cold	cc. of $N/10$ potassium permanganate reduced by 5 cc. phloridzin urine	cc. of $N/10$ potassium permanganate reduced by 5 cc. normal urine	% sugar by Bertrand's method	% sugar as calculated polarimetrically immediately after urine passed, and at 24 hourly intervals
1	No change	0·5	0·4	1·75	1·6
2	,,	0·55	0·75	3·62	3·35 3·37
3	,,	0·6	0·5	1·33	1·18
4	,,	0·5	0·6	1·45	1·06 1·08 1·08 1·06
5	,,	0·65	0·6	0·7	—
6	,,	0·5	0·6	1·72	1·60 1·63 1·81
7	,,	0·5	0·6	4·5	4·14 4·11 4·19
8	,,	0·5	0·5	2·84	2·44 2·47 2·47
9	,,	0·7	0·6	3·1	2·9 2·83 2·92
10	,,	0·6	0·6	2·2	1·8 1·71 1·9

Conclusions.

(1) The sugar excreted in the urine of the normal subject, as the result of an injection of phloridzin, is not γ-glucose but appears to be the ordinary α-β-variety.

(2) Since no evidence of the presence of γ-glucose was obtained in the urine of healthy individuals, it is unlikely that this form of sugar is the variety present in normal blood.

(3) The sugar passed by the diabetic subject, and that passed by the normal individual as the result of the use of phloridzin, appear to be of the same nature.

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LXXIX. STUDIES IN THE SULPHUR METABOLISM OF THE DOG. II. THE CONSTANCY OF THE RELATIVE OUTPUT OF ETHEREAL SULPHATE AND OF NEUTRAL SULPHUR AFTER THE ORAL ADMINISTRATION OF THE HALOGEN SUBSTITUTED BENZENES.

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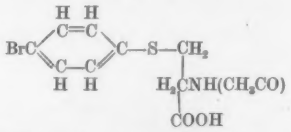
(*Received April 14th, 1924.*)

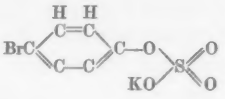
Baumann and Preusse [1879] were the first to point out that bromobenzene was partly excreted by the dog as *p*-bromophenylmercapturic acid and partly as *p*-bromophenyl sulphate. It is known that *p*-bromophenol gives rise to *p*-bromophenyl sulphate, but as yet no mercapturic acid has been isolated from the urine of dogs taking this substance. It is largely a question of dosage. The phenol coefficient (*B. coli*) for *p*-bromophenol is 7·4 [Cooper and Woodhouse, 1923] and its toxicity to mice is about the same as that of phenol [Bechold and Ehrlich, 1906]. On the other hand bromobenzene can be administered safely to animals in comparatively large doses. To prepare mercapturic acids therefore the benzenes and not the phenols have always been administered to dogs. Observers generally do not make a distinction between the metabolism of bromobenzene and that of *p*-bromophenol. They assume that bromobenzene is first oxidised to *p*-bromophenol before it is converted to mercapturic acid. This is almost certainly true but the proof is incomplete. In other directions also our knowledge is very incomplete. The evidence for the synthesis of a mercapturic acid from any substance other than a monohalogen benzene or by any animal other than the dog is not definite, though there is presumptive evidence that the monohalogen naphthalenes also give rise to mercapturic acids in the dog [Baumann, 1883] and that the rabbit can synthesise a mercapturic acid from bromobenzene [Rhode, 1922].

It is this lack of knowledge that has tended to make writers draw a line of distinction between the metabolism of the monohalogen benzenes and the metabolism of all other benzene derivatives in the dog; and a line of distinction between the behaviour of the dog and the behaviour of all other species towards the monohalogen benzenes. Reference to their papers shows that such distinctions were not in the minds of Baumann and Preusse [1879]

or of Jaffe [1879], who shares with Baumann and Preusse the credit of the discovery of the mercapturic acids. Hopkins [1907] and Sherwin [1922] have both suggested that mercapturic acid is an intermediary stage in the formation of ethereal sulphate and that the addition of the halogen to the benzene ring prevents the further oxidation of the intermediary mercapturic acid in the dog but not in other species. It is to be noted however that the structure of the two types of compound is entirely different and that the change from one to the other is not a simple oxidation.



p-Bromophenylmercapturic acid



Potassium *p*-bromophenyl sulphate

Such a theory is put forward to account for the occurrence of a remarkable synthesis, excited by a substance which the animal would never meet under natural conditions. It is surely much easier to assume that many substances of common nature are excreted as mercapturic acids, the isolation of which is not readily achieved, and that bromobenzene merely follows a general line of metabolism.

Muldoon, Shiple and Sherwin [1923, 1] have recently failed to find any animal, other than the dog, capable of synthesising mercapturic acid from bromobenzene. They failed also to demonstrate in the pig the direct synthesis of ethereal sulphate from sodium sulphate and either phenol, *p*-chlorophenol or bromobenzene. On the other hand the increase in ethereal sulphate after *p*-bromophenylmercapturic acid was not sufficient to prove it to be an intermediary product of metabolism. Presumably no toxic signs were observed after mercapturic acid, such as has been found by Hele and Callow in the dog [1924]. The full account of their work is awaited with interest.

It will be shown in this paper that chlorobenzene like guaiacol [Hele, 1924] may be excreted in combination with preformed sulphate added to the diet. This at once renders an intermediary stage unnecessary though it does not preclude the possible oxidation of a mercapturic acid to ethereal sulphate. In the next paper of this series there will be discussed the possibility of the excretion by the dog of mercapturic acids other than those already known [Hele and Callow, 1923].

Until accurate methods of estimating mercapturic acids are discovered it is necessary to rely on sulphur analyses of the urine for quantitative studies. This is unsatisfactory. Zeller and Straczewski [1914] and Kapfhammer [1921] obtained a rise in the neutral sulphur of the urine after giving bromobenzene by mouth to dogs on a protein-free diet and yet were unable to isolate a mercapturic acid. In the course however of the present investigations a quantitative relation was established between the output of ethereal sulphate and of neutral sulphur that would suggest that chlorobenzene given to a dog on

a standard mixed diet, containing protein, appears in the urine in only two sulphur combinations, as ethereal sulphate and as mercapturic acid. Whether this is true or not (and proof will only be forthcoming when accurate analyses of the several substances comprised in neutral sulphur are possible), it is certain that the dog reacts towards bromobenzene and chlorobenzene in a very constant fashion and that the quantitative effect on the sulphur partition in the urine can within limits be predicted for any given dose. For the sake of brevity it will be assumed in this paper that the increase in the output of neutral sulphur in the urine over the normal is due entirely to mercapturic acid but it must not be forgotten that the increase may be due to two or more sulphur-containing bodies.

Experimental.

The work described in this paper was commenced in the year 1914. It was found in the course of preparing mercapturic acid from the dog that the simultaneous administration of cystine seemed to increase the yield. In consequence quantitative experiments (Nos. 1, 2, 3) to show the direct union of bromobenzene and cystine, both given by the mouth, were carried out with apparently satisfactory results, though subsequently the experiments were proved fallacious. In 1920 this question was reopened (Nos. 4, 5, 6) but no proof of such direct union was obtained. On the other hand the constant quantitative character of the reaction of the dog to chlorobenzene became apparent and two experiments (Nos. 7, 8) were carried out to test this point more completely. Subsequently in 1921–22 four experiments (Nos. 9, 10, 11, 12) were carried out, which showed that chlorobenzene will react with sodium sulphate and with cystine without materially upsetting the quantitative relation between the amount of ethereal sulphate and the amount of mercapturic acid produced. The paper therefore falls into three parts: (1) early experiments, (2) experiments exhibiting a constant quantitative relation between ethereal sulphate and neutral sulphur, and (3) experiments exhibiting the union of administered sulphate and of administered cystine with the oxidation products of chlorobenzene.

Bitches, rendered easy of catheterisation by operation, were used for the experiments. The standard diets were composed of lean beef, suet, arrowroot biscuits and milk. The food was given at 9.30 a.m. and 5 p.m. The daily allowance was divided equally between the two meals except in the early part of Exp. 5. The monobromobenzene or monochlorobenzene was given in gelatin capsules, usually in a single dose with the morning meal. In the experiments in which these substances were given over several days the daily dose was divided between the morning and evening meals. The sodium sulphate and cystine were also given by the mouth and when given were administered at each meal. After the animal had been on a standard diet for four or five days the urine was collected every 24 hours, the animal being catheterised at least once a day just before the morning meal, when the

24 hours' urine was made up. Total nitrogen, total sulphur, total sulphate and inorganic sulphate were estimated in the urine and sometimes free and combined phenols, ammonia and nitrogen titratable in presence of formaldehyde. Ethereal sulphate was estimated by deducting inorganic sulphate from total sulphate, and neutral sulphur by deducting total sulphate sulphur from total sulphur. Total nitrogen, total sulphur and sometimes free phenols were estimated in the faeces.

For all the sulphur estimations Folin's methods [1905] were employed. For total sulphate and for inorganic sulphate these methods have become general. The methods are not unduly long and more accurate than any benzidine method. If duplicates did not agree within 1 %, further estimations were carried out. For total sulphur estimations Folin's technique by oxidation with sodium peroxide was preferred to any other method. The accuracy of the method has been well established by Folin. An agreement within 3 % between duplicates was regarded as sufficient save on important days. A spirit lamp was always used for the fusion of the peroxide. For filtration Gooch crucibles were used throughout and it was found that, if they were properly packed with asbestos, the filtration and washing of the barium sulphate precipitates did not take more than eight minutes for each estimation. About a litre of hot water for washing was used for each of the total sulphur estimations and half that quantity for each of the other estimations. The precipitates were washed until the washings were chloride-free. No essential departures were made from the methods as described by Folin.

For the estimation of phenols the method of Folin and Denis [1915, 1916] was used. Though open to certain objections that have been met in Tisdall's modification [1920], it was found to be more convenient than the latter method. As these estimations were not considered an essential part of the experiments, accuracy was allowed to give place to convenience.

For the estimation of total nitrogen and of total sulphur in the faeces, it was found better not to dry them. The faeces were rubbed up in a mortar with a quantity of water sufficient to make an emulsion as far as possible uniform. 25 to 50 cc. were taken for estimations of nitrogen or sulphur. For estimating sulphur Folin's technique was employed but with twice the quantity of sodium peroxide (20 g.).

The estimation of total sulphur in the food was not so easy. The food sample was weighed into the nickel crucible, containing 10 cc. of water and 3 g. sodium peroxide. The contents were evaporated to dryness on the water-bath and then heated for ten minutes. The crucible was allowed to cool, 1 cc. water poured in and then 17 g. sodium peroxide were sprinkled over the contents. The subsequent procedure was identical with that given in Folin's paper. The method was entirely satisfactory for meat and milk, but for the biscuit, containing large amounts of carbohydrate and but little sulphur, there was the almost certain risk of explosion. To prevent this it was found necessary to burn off most of the carbon in the preliminary heating before

fusion, the substance having been made strongly alkaline in the earlier stage. In this manner the method became moderately satisfactory but it is not suggested that the sulphur analyses for the biscuit are anything but approximate. Eight samples of each foodstuff were taken and estimations carried out in duplicate. The mean values obtained are shown in the following table. Figures in brackets are calculated from the tables of Attwater and Bryant [1899].

Table I.

	Total N %	Total S %	Calories K per 100 g.
Beef (lean shank)	3·41	0·226	132·6
Milk	0·51	0·044	(71·8)
Suet	(0·75)	(0·05)	(780·4)
Biscuit	1·10	0·127	605·7

In the tables of urine analysis total sulphur, total sulphate, ethereal sulphate and neutral sulphur are expressed in grams sulphur. The relation between total sulphur and total nitrogen is expressed as the ratio of the sulphur multiplied by 100 to nitrogen and not in the reverse manner, as the sulphur output is the more directly influenced by the compounds given. The relation between ethereal sulphate and neutral sulphur $\left(\frac{E}{E+NS}\right)$ is expressed as the ratio multiplied by 100 of the excess ethereal sulphate to the sum of the excess ethereal sulphate and excess neutral sulphur. To give an example: for the 14 normal days of Exp. 4 the average output of ethereal sulphate is 0·007 g. S and of neutral sulphur 0·074 g. S. On August 12 the ethereal sulphate is 0·047 g. S and neutral sulphur 0·120 g. S. The excess figures are respectively 0·040 and 0·046 g. S. The sum of these is 0·086 g. S and the ratio $\frac{E}{E+NS}$ is 46·5. In effect the figure represents the percentage excreted as ethereal sulphate of the bromo- or chloro-benzene in combination with sulphur, as in each group one molecule of the benzene corresponds to one atom of sulphur.

Free and total phenols are expressed as grams phenol. The method of Folin and Denis does not exclude hydroxy-acids, and there is little doubt that the high value for free phenols on the normal days is due to hydroxy-acids. After chlorobenzene therefore the rise in conjugated phenols appears to be out of proportion to the rise in free phenols. If these figures are treated in a manner similar to the treatment accorded to the sulphur figures, there is also found to be a constancy. The phenol ratio shown in the tables is the ratio multiplied by 100 of the excess free phenol over the normal to the excess total phenol over the normal.

In accordance with the observations of Folin and Denis [1916] no conjugated phenols were found in the faeces. There was no appreciable rise of free phenols in the faeces after the administration of chlorobenzene.

Ammonia (Folin) and "formal" nitrogen (Sörensen) were estimated in the urine on days on which the output of nitrogen was markedly high. This was

done to discover whether there was any increased output of amino-acid nitrogen. In no case however was there any such indication. The analyses have not been shown.

The mean normal daily output of each group of substances analysed is shown in the tables and the probable error for each mean, calculated by the method of mean squares. The probable error has also been determined for the ratios by compounding in a similar manner the errors on the figures used in the calculations. The analytical error (1 to 3 %) on each estimation has not however been taken into account. The probable error is large with the smaller doses of chlorobenzene and on the third and subsequent days after larger doses.

Some tables are given in full. Exp. 4 may be taken as a typical example. Others have been summarised to save space. In the summarised tables, days following the experimental days, during which the animal was returning to the normal, are not shown.

(1) Early Experiments.

The chief difficulty experienced was the maintenance of the dogs in health. Confinement to the metabolism cages increased this difficulty but the main cause was the use of too large doses of bromobenzene, which caused loss of appetite.

Exp. 1. "Judy," May 24 to June 2, 1914. The dog was given a diet of meat and fat and received by mouth over a period of five days bromobenzene, 23 g., and cystine, 18·5 g.

Exp. 2. "Judy," June 17 to 30, 1914. The dog refused to take any fat and was given therefore only meat. Bromobenzene, 15 g., was given by mouth over a period of four days (June 19 to 22).

The urine analysis for the first six days is given in Table II.

Table II.

Date 1914	Vol. cc.	T. N g. N	T. S g. S	In. SO_4 g. S	Eth. SO_4 g. S	N. S g. S	$\frac{E}{E+NS}$
June 17	210	3·08	0·164	0·079	0·015	0·070	—
„ 18	160	3·31	0·174	0·101	0·013	0·060	—
„ 19	280	4·12	0·313	0·083	0·083	0·147	(45·7)
„ 20	310	6·06	0·432	0·000	0·123	0·309	(30·9)
„ 21	260	6·35	0·403	0·000	0·073	0·330	(18·2)
„ 22	410	6·64	0·392	0·000	0·089	0·303	(24·0)

The animal's weight fell from 8·17 to 6·60 kilo. in 14 days.
Nitrogen balance −45·70 g.; sulphur balance −2·345 g.

The output of neutral sulphur in the first experiment was very much greater than in the second and the output of nitrogen much less, suggesting that the cystine in the first experiment had united with some of the bromobenzene and prevented tissue breakdown. This was probably true but the results are vitiated by two considerations. The high nitrogen output in the second experiment is explained by the refusal of the dog to take the fat. The animal was being starved. The high value for neutral sulphur in the

first experiment is partly due to unoxidised products of bacterial decomposition of the cystine in the intestine. Exp. 2 does however offer confirmatory evidence to Exp. 10 and is therefore shown.

Exp. 3. "Doris," August 3 to 15, 1914. The diet was adequate but could not be maintained to the end of the experiment. It consisted of meat, biscuit and fat. Cystine, 2 g., was given daily from August 6 to 12 inclusive and bromobenzene, 5 g. daily, on August 7, 8, 9.

The urine analysis may be summarised as follows:

Table III.

Date 1914	Vol. cc.	T. N g. N	T. S g. S	In. SO_4 g. S	Eth. SO_4 g. S	N. S g. S
Control period (average six days)	141	3·65	0·193	0·118	0·009	0·066
Probable error	± 45	0·73	0·035	0·033	0·003	0·010
August 7	156	3·98	0·479	0·000	0·147	0·332
„ 8	270	4·52	0·645	0·000	0·194	0·451
„ 9	300	4·69	0·591	0·000	0·190	0·401

The animal's weight fell from 5·63 to 4·90 kilo. in 13 days.
Nitrogen balance −4·57 g.; sulphur balance +1·706 g.

This experiment resembles and confirms Exp. 9. There is however no adequate means of checking the rise in neutral sulphur due to the effect of the cystine alone. It is clear that some of the cystine has been utilised to form ethereal sulphate. It is not however clear if any of it has been utilised to form mercapturic acid.

(2) Experiments exhibiting a constant quantitative relation between ethereal sulphate and neutral sulphur.

In all these experiments and in those of the later group an adequate diet was maintained throughout. To keep the animals in a happy normal condition they were allowed more liberty. They were placed in the metabolism cages only at night. During the day they were chained to the laboratory bench and catheterised at 3, 4, or 5 hour intervals according to their habit. In this way long experiments could be continued without forcible feeding, which was always unsatisfactory. It was rare for an experiment to be discontinued suddenly on account of loss of urine or failure on the part of the dog to digest its food.

Exp. 4. "Phoena," August 8 to 31, 1920 (Table IV).

This experiment was devised to show the union between chlorobenzene and sodium sulphate both given by mouth, in the same way in which guaiacol may be shown to unite with sodium sulphate. Trial doses of chlorobenzene, 0·5 cc. or 0·55 g. and 3 cc. or 3·33 g., were given and it was then observed for the first time that the relative amounts of "extra" ethereal sulphate and of "extra" neutral sulphur were the same in the two cases within the error of the experiment. When however an attempt was made (August 23–26) to link up the chlorobenzene with sodium sulphate, the result was entirely negative. No additional ethereal sulphate was formed and the ratio $\frac{E}{E+NS}$ did not change.

Table IV. (Exp. 4.)

Diet			T. N g.	T. S g.	Calories K
Lean meat	75 g.	...	2·56	0·169	100
Suet	15 g.	...	0·11	0·007	117
Biscuit	60 g.	...	0·66	0·076	363
Milk	160 cc.	...	0·83	0·074	115
		Total...	4·16	0·326	695

Sodium sulphate, cryst.: 3 g. daily by mouth from August 23 to 26.
Chlorobenzene by mouth at 9.30 a.m.: 0·55 g. on August 12 and 3·33 g. on August 16 and 24.

	Urine											Faeces			
							Phenols								
Date 1920	Vol. cc.	T. N g. N	T. S g. S	In. SO_4 g. S	Eth. SO_4 g. S	N. S g. S	Free g. phenol	Conjugated g. phenol	T. S T. N	E / E+NS	Phenol ratio	T. N g. N	T. S g. S	Free phenols g. phenol	Percent. of dose chlorobenzene excreted with S
Aug. 8	273	3·60	0·183	0·102	0·006	0·075	0·062	0·006	5·08	Normal day		—	—	—	—
,, 9	260	3·48	0·180	0·108	0·006	0·066	0·054	0·012	5·17	,,		0·45	0·093	0·060	—
,, 10	270	3·79	0·198	0·121	0·007	0·070	0·056	0·013	5·22	,,		0·33	0·067	0·045	—
,, 11	268	3·80	0·206	0·119	0·009	0·078	0·056	0·011	5·42	,,		—	—	—	—
,, 12	255	3·97	0·219	0·050	0·047	0·120	0·118	0·107	5·51	46·5±4·0	40·9± 2·9	0·50	0·107	0·066	62·0
,, 13	233	4·30	0·222	0·125	0·013	0·084	0·075	0·030	5·16	—	—	—	—	—	—
,, 14	238	4·27	0·226	0·135	0·009	0·082	0·067	0·017	5·29	Normal day		0·42	0·088	0·064	—
,, 15	243	3·99	0·218	0·131	0·008	0·079	0·060	0·015	5·46	,,		—	—	—	—
,, 16	240	4·75	0·289	0·005	0·123	0·261	0·215	0·325	8·19	38·3±1·0	34·1± 1·0	0·35	0·074	0·049	
,, 17	255	4·73	0·194	0·000	0·060	0·134	0·141	0·155	4·10	46·9±3·0	38·2± 2·2	0·33	0·069	0·059	49·5
,, 18	275	3·94	0·134	0·000	0·023	0·111	0·075	0·058	3·40	30·2±4·8	32·8± 7·4	0·30	0·065	0·047	
,, 19	243	3·70	0·208	0·102	0·006	0·100	0·063	0·024	5·62	—	—	—	—	—	—
,, 20	250	3·98	0·230	0·137	0·009	0·084	0·063	0·019	5·78	Normal day		0·49	0·091	0·076	—
,, 21	265	4·10	0·213	0·137	0 005	0·071	0·052	0·005	5·19	,,		0·38	0·083	0·054	—
,, 22	282	3·82	0·228	0·134	0·011	0·083	0·050	0·009	5·96	,,		—	—	—	—
,, 23	275	4·52	0·495	0 411	0·006	0·078	0·054	0·008	10·96	—	—	0·42	0·074	0·056	—
,, 24	368	4·96	0·606	9·222	0·131	0·253	0·233	0·299	12·22	40·9±1·0	38·6± 1·0	0·16	0·049	0·023	
,, 25	335	4·41	0·442	0·212	0·073	0·157	0·136	0·129	10·02	44·3±2·2	41·7± 2·3	0·37	0·108	0·061	55·2
,, 26	285	3·67	0·440	0·288	0·037	0·115	0·067	0·045	12·00	42·2±4·5	30·4±11·1	0·32	0·085	0·047	
,, 27	330	3·64	0·182	0·093	0·011	0·078	0·053	0·025	5·00	Normal day		0·32	0·061	0·043	—
,, 28	200	3·30	0·165	0·094	0·006	0·065	0·041	0·015	5·00	,,		0·29	0·058	0·040	—
,, 29	257	3·80	0·192	0·123	0·004	0·065	0·043	0·010	5·05	,,		0·31	0·051	0·047	—
,, 30	350	3·89	0·198	0·124	0·007	0·067	0·045	0·015	5·09	,,		0·29	0·059	0·046	—
,, 31	273	4·01	0·211	0·138	0·008	0·065	0·045	0·013	5·26	,,		0·27	0·060	0·043	—
Normal output (av. 5 days)	268	3·82	0·202	0·121	0·007	0·074	0·053	0·013	5·28	—	—	—	—	—	—
Variable for	± 35	0·24	0·019	0·015	0·002	0·007	0·007	0·005	0·27	—	—	—	—	—	—

The dog's weight fell from 7·26 to 7·12 kilo. in 24 days.

Balance Sheet (24 days).

	T. N g.	T. S g.	T. N g.	T. S g.
Excreted in urine	96·42	6·179		
,, faeces	6·30	1·342		
			102·72	7·521
Given by mouth, food	99·84	7·824		
,, ,, sodium sulphate	—	1·192		
			99·84	9·016
Balance			−2·88	+1·495

Exp. 5. "Phoena," Sept. 7 to Oct. 3, 1920 (Table V).

It was felt that perhaps the failure in the previous experiment to show the direct union of sodium sulphate with chlorobenzene was due to the fact that the sodium sulphate was absorbed and excreted by the kidney before the chlorobenzene could be oxidised to chlorophenol and therefore could not upset the balance at the seat of synthesis. In the fifth experiment the sodium sulphate was given in small doses every hour throughout the day. The sulphate had however no effect on the ethereal sulphate or on the $\frac{E}{E+NS}$ ratio. Two attempts were made to show the direct union of cystine and chlorobenzene

Table V. (Exp. 5.)

Diet as in Exp. 4.

From Sept. 6 to 14, inclusive, the food was given thus:

Meat: 10 g. at 10.30 a.m., 25 g. at 12.30 p.m., 40 g. at 6.30 p.m.
Biscuit: 30 g. at 9.30 a.m. and at 6.30 p.m.
Suet: 10 g. at 12.30 p.m., 5 g. at 6.30 p.m.
Milk: 20 cc. every hour from 9.30 a.m. to 4.30 p.m.

Sodium sulphate, cryst.: 0·4 g. with each feed of milk, 16 cc. daily = 0·32 g. S on Sept. 9, 10, 11.
Cystine by mouth: 1·5 g. on Sept. 17, 25, 26, 27.
Chlorobenzene by mouth: 1·11 g. at 10.30 a.m. and 2·22 g. at 12.30 p.m. on Sept. 11; 3·33 g. at 9.30 a.m. on Sept. 17 and on Sept. 26.

	Urine											
							Phenols					
Date 1920	Vol. cc.	T. N g. N	T. S g. S	In. SO_4 g. S	Eth. SO_4 g. S	N. S g. S	Free g. phenol	Conjugated g. phenol	T. S / T. N	K / E+NS	Phenol ratio	Percent. of dose chlorobenzene excreted with S
Mean normal output (av. 13 days)	287	3·93	0·213	0·124	0·006	0·083	0·050	0·014	5·42	—	—	—
Probable error ±	30	0·19	0·021	0·017	0·002	0·007	0·006	0·002	0·37	—	—	—
Sept. 11	345	4·91	0·640	0·218	0 149	0·273	0·203	0·309	13·03	42·9±1·0	34·1±0·9	
„ 12	273	4·42	0·450	0·198	0·066	0·186	0·174	0·149	10·18	36·8±1·7	47·9±1·3	62·9
„ 13	240	3·82	0·164	0·014	0·031	0·119	0·097	0·071	4·29	41·0±5·1	45·2±3·3	
„ 17	372	4·07	0·535	0·080	0·136	0·319	0·258	0·301	13·14	—	42·0±0·7	50·4
„ 18	258	4·08	0·204	0·001	0·063	0·140	0·145	0·140	5·00	—	43·0±1·6	
„ 26	278	4·74	0·630	0·176	0·130	0·324	0·258	0·293	13·29	—	42·7±0·7	
„ 27	255	4·30	0·474	0·196	0·066	0·212	0·140	0·148	11·03	—	40·2±1·6	68·6
„ 28	255	4·06	0·245	0·086	0·028	0·131	0·078	0·058	6·04	—	38·8±5·2	

The dog's weight fell from 7·48 to 6·96 kilo. in 27 days.

Balance Sheet (27 days).

	T. N g.	T. S g.	T. N g.	T. S g.
Excreted in urine	108·85	7·838		
„ faeces	7·89	1·804		
			116·74	9·642
Given by mouth, food	112·32	8·802		
„ „ S compounds	0·70	2·560		
			113·02	11·362
		Balance	−3·72	+1·720

Table VI. (Exp. 6.)

Diet:—Meat, 300 g.; suet, 20 g.; biscuit, 15 g.; T. N, 10·54 g.; T. S, 0·707 g.; cal., 645 K.

From Nov. 10 diet irregular.

Chlorobenzene 3·33 g. given by mouth at 9.30 a.m. on Nov. 9.

	Urine									
							Phenols			
Date 1920	Vol. cc.	T. N g. N	T. S g. S	In. SO_4 g. S	Eth. SO_4 g. S	N. S g. S	Free g. phenol	Conjugated g. phenol	T. S / T. N	Phenol ratio
Mean normal output (av. 7 days)	294	8·21	0·449	0·284	0·012	0·153	0·074	0·007	5·47	—
Probable error ±	15	0·44	0·034	0·019	0·004	0·030	0·005	0·002	0·30	—
Nov. 9	305	8·85	0·524	0·042	0·136	0·346	0·256	0·295	5·92	33·7±0·8
„ 10	300	6·35	0·313	0·046	0·061	0·206	0·144	0·129	4·93	36·4±1·7

The dog's weight fell from 6·75 to 6·49 kilo. in 12 days.

Balance Sheet (12 days).

	T. N g.	T. S g.	T. N g.	T. S g.
Excreted in urine	85·15	4·709		
„ faeces	3·62	0·714		
			88·77	5·423
Given by mouth, food			98·11	6·665
		Balance	+9·34	+1·242

both given by mouth. These experiments were vitiated by the failure to appreciate the effect of the daily dose (1·5 g.) of cystine on the neutral sulphur output. In consequence the $\frac{E}{E+NS}$ ratio has not been calculated for these days. The phenol ratio is similar to that in Exp. 4.

Exp. 6. "Phoena," November 2 to 13, 1920 (Table VI).

In this experiment the dog was placed on a high protein diet. It was considered that perhaps protein might be more effective than cystine in the synthesis of mercapturic acid. The absorption of cystine from the intestine under these circumstances would not be so rapid and consequently there would be a greater likelihood of maintaining over a long period an increase in the concentration of cystine at the seat of synthesis. The dog refused to take so large an amount of meat before the experiment was completed. Apart from this failure however the variations in neutral sulphur output (from 0·150 to 0·196 g.) were very extreme, so that no average normal value could be determined. It is interesting to note that doubling the protein of the

Exp. 7. "Sophia" June 29 to July 17, 1921.

Table VII. (Exp. 7.)

Diet			T. N g.	T. S g.	Calories K
Lean meat	75 g.	...	2·56	0·169	100
Suet	10 g.	...	0·07	0·005	78
Biscuit	60 g.	...	0·66	0·076	363
Milk	150 cc.	...	0·78	0·069	108
	Total	...	4·07	0·309	649

Date 1921	Oral dose bromo-benzene g.	Urine: Vol. cc.	T. N g. N	T. S g. S	In. SO_4 g. S	Eth. SO_4 g. S	N. S g. S	T. S T. N	E / E+NS	Faeces: T. N g. N	Faeces: T. S g. S	Percent. of dose bromo-benzene excreted with S
June 29	—	204	3·74	0·146	0·096	0·007	0·043	3·91	Normal day	—	—	—
,, 30	—	204	3·72	0·123	0·084	0·004	0·035	3·31	,,	0·83	0·128	—
July 1	—	206	3·26	0·127	0·085	0·003	0·039	3·90	,,	—	—	—
,, 2	—	188	3·58	0·150	0·106	0·005	0·039	4·19	,,	—	—	—
,, 3	0·5	210	3·90	0·167	0·040	0·035	0·092	4·28	38·1 ± 3·4	—	—	74·5
,, 4	—	212	3·50	0·140	0·082	0·007	0·051	4·00	Normal day	—	—	—
,, 5	—	246	3·67	0·179	0·126	0·009	0·044	4·88	,,	0·71	0·107	—
,, 6	—	228	3·87	0·179	0·130	0·008	0·041	4·63	,,	—	—	—
,, 7	3·0	290	4·85	0·351	0·008	0·114	0·229	7·24	37·0 ± 0·9	—	—	
,, 8	—	154	4·37	0·175	0·000	0·046	0·129	4·00	32·2 ± 1·9	—	—	75·2
,, 9	—	124	3·86	0·110	0·015	0·032	0·063	2·85	,,	0·77	0·129	
,, 10	—	148	3·92	0·193	0·130	0·005	0·058	4·92	Normal day	—	—	—
,, 11	—	136	3·97	0·202	0·142	0·005	0·055	5·10	,,	—	—	—
,, 12	0·75	176	4·20	0·240	0·055	0·044	0·141	5·72	28·4 ± 1·7	—	—	87·6
,, 13	—	150	3·88	0·171	0·091	0·011	0·069	4·41	,,	—	—	—
,, 14	—	132	3·47	0·151	0·102	0·008	0·041	4·35	Normal day	1·11	0·149	—
,, 15	0·37	205	3·65	0·186	0·064	0·024	0·098	5·10	25·3 ± 3·0	—	—	93·4
,, 16	—	210	3·38	0·160	0·108	0·008	0·044	4·73	Normal day	—	—	—
,, 17	—	132	3·70	0·186	0·133	0·007	0·046	5·02	,,	0·74	0·133	—
Mean normal output (av. 12 days)	—	187	3·65	0·161	0·110	0·006	0·045	4·41	—	—	—	—
Probable error	— ±	40	0·21	0·025	0·020	0·002	0·006	0·53	—	—	—	—

All doses of bromobenzene were given by mouth at 9.30 a.m.
The dog's weight fell from 5·91 to 5·82 kilo. in 19 days.

Balance Sheet (19 days).

	T. N g.	T. S g.	T. N g.	T. S g.
Excreted in urine	72·49	3·336		
,, faeces	4·16	0·646		
			76·65	3·982
Given by mouth, food			77·33	6·061
		Balance	+0·68	+2·079

food doubled the neutral sulphur output. There was no indication that there had been any greater production of mercapturic acid. The phenol output, though larger than in the last experiment, did not show large variations, so that an average normal output could be determined. The phenol ratio was similar to that in the previous experiments.

Exp. 8. "Phoena," August 11 to September 2, 1921.

Table VIII. (Exp. 8.)

Diet as in Exp. 4.

Date 1921	Oral dose chloro-benzene g.	Urine: Vol. cc.	T. N g. N	T. S g. S	In. SO_4 g. S	Eth. SO_4 g. S	N. S g. S	Phenols: Free g. phenol	Phenols: Conjugated g. phenol	T. S T. N	$\frac{E}{E+NS}$	Phenol ratio	Faeces: T. N g. N	Faeces: T. S g. S	Percentage dose chlorobenzene excreted with
Aug. 11	—	164	3·90	0·197	0·133	0·010	0·054	0·045	0·014	5·05	Normal day		—	—	—
,, 12	—	196	4·20	0·215	0·147	0·007	0·061	0·041	0·012	5·12	,,		0·28	0·052	—
,, 13	—	204	4·03	0·195	0·132	0·006	0·057	0·041	0·012	4·84	,,		0·43	0·098	—
,, 14	—	223	4·26	0·204	0·138	0·004	0·062	0·041	0·012	4·79			—	—	—
,, 15	0·37	190	4·10	0·205	0·079	0·030	0·096	0·069	0·078	5·00	36·6±4·2	27·9±4·5	0·48	0·109	57·4
,, 16	—	178	4·05	0·209	0·133	0·007	0·069	0·039	0·021	5·16	Normal day		—	—	—
,, 17	0·28	184	3·89	0·209	0·080	0·028	0·101	0·053	0·071	5·38	31·7±3·7	12·7±7·0	0·54	0·120	79·4
,, 18	—	166	3·91	0·203	0·127	0·012	0·064	0·047	0·025	5·20	Normal day		—	—	—
,, 19	—	194	4·26	0·218	0·142	0·008	0·068	0·051	0·010	5·12			0·41	0·088	—
,, 20	2·22	196	4·79	0·344	0·019	0·112	0·213	0·153	0·252	7·18	40·2±1·0	31·4±1·1	—	—	
,, 21	—	200	4·38	0·155	0·000	0·044	0·111	0·105	0·105	3·54	40·5±3·0	40·2±2·4	—	—	59·0
,, 22	—	170	4·22	0·159	0·070	0·020	0·069	0·069	0·044	3·77	—	—	0·60	0·127	
,, 23	—	174	4·46	0·195	0·127	0·009	0·059	0·046	0·031	4·37	Normal day		—	—	—
,, 24	—	238	4·50	0·204	0·145	0·008	0·051	0·041	0·021	4·53			0·48	0·096	—
,, 25	0·55	228	4·36	0·254	0·086	0·046	0·122	0·082	0·104	5·83	37·3±2·5	29·6±3·1	—	—	
,, 26	—	198	4·18	0·205	0·116	0·018	0·071	0·046	0·034	4·91	—	—	0·48	0·108	79·1
,, 27	—	162	4·39	0·216	0·147	0·010	0·059	0·036	0·022	4·92	Normal day		—	—	—
,, 28	2·22	173	4·66	0·354	0·028	0·109	0·217	0·152	0·248	7·60	38·8±1·0	31·5±1·1	0·42	0·087	
,, 29	—	180	4·46	0·174	0·000	0·046	0·128	0·091	0·109	3·90	35·2±2·3	33·1±2·7	—	—	62·1
,, 30	—	140	3·96	0·145	0·054	0·020	0·071	0·056	0·052	3·66	—	—	0·52	0·113	
,, 31	—	162	4·13	0·191	0·122	0·013	0·056	0·052	0·033	4·63	Normal day		—	—	—
Sept. 1	—	162	4·18	0·198	0·143	0·007	0·048	0·052	0·013	4·74	,,		—	—	—
,, 2	—	132	4·27	0·209	0·152	0·008	0·049	0·050	0·018	4·90	,,		0·52	0·117	—
Mean normal output (av. 13 days)		176	4·20	0·204	0·138	0·008	0·058	0·045	0·016	4·88	—	—	—	—	—
Probable error	±	15	0·18	0·008	0·008	0·002	0·006	0·005	0·005	0·25	—	—	—	—	—

All doses of chlorobenzene were given by mouth at 9.30 a.m. save on August 28, on which day 1·11 g. was given at 9.30 a.m. and 1·11 g. at 5.30 p.m.
The dog's weight fell from 8·10 to 7·94 kilo. in 23 days.

Balance Sheet (23 days).

	T. N g.	T. S g.	T. N g.	T. S g.
Excreted in urine ...	97·54	4·858		
,, faeces ...	5·16	1·115		
			102·70	5·973
Given by mouth, food ...			95·68	7·498
			Balance −7·02	+1·525

Exps. 7 and 8 were carried out to determine whether the ratios were affected by the size of dose or not. Equivalent doses of bromobenzene and of chlorobenzene were given by mouth to two dogs on standard diets. 1 cc. or 1·5 g. bromobenzene is equivalent to 1 cc. or 1·11 g. chlorobenzene. The ratios are similar throughout, though the values for the $\frac{E}{E+NS}$ ratio are rather lower in Exp. 7 than in Exp. 8. This may be due to a difference in the dog or to a difference in the compound administered. The differences are not however great except for the small doses, where the experimental error is largest.

(3) EXPERIMENTS EXHIBITING THE UNION OF ADMINISTERED SULPHATE AND OF ADMINISTERED CYSTINE WITH THE OXIDATION PRODUCTS OF CHLOROBENZENE.

In these experiments the same dog and the same standard diet were used throughout.

Exp. 9. "Vixen," November 19 to December 17, 1921.

Table IX. (Exp. 9.)

Diet			T. N g.	T. S g.	Calories K
Lean meat	80 g.	...	2·73	0·181	106
Suet	20 g.	...	0·15	0·010	156
Biscuit	60 g.	...	0·66	0·076	363
Milk	160 cc.	...	0·83	0·074	115
		Total...	4·37	0·341	740

Date 1921	Chloro-benzene by mouth g.	Cystine by mouth g.	Urine Vol. cc.	T. N g. N	T. S g. S	In. SO_4 g. S	Eth. SO_4 g. S	N. S g. S	T. S / T. N	E / E+NS	Faeces T. N g. N.	Faeces T. S g. S	Percent. of dose chloro-benzene excreted with S
Nov. 19	—	—	244	4·11	0·240	0·128	0·006	0·106	5·84	Normal day	—	—	—
,, 20	—	—	212	4·35	0·253	0·146	0·010	0·097	5·81	,,	—	—	—
,, 21	—	—	188	3·92	0·223	0·135	0·009	0·079	5·68	,,	—	—	—
,, 22	—	1	182	3·95	0·390	0·236	0·007	0·097	9·88	—	—	—	—
,, 23	—	1	186	4·33	0·482	0·344	0·006	0·132	11·13	—	—	—	—
,, 24	—	1	188	4·18	0·466	0·323	0·010	0·133	11·15	—	1·31	0·189	—
,, 25	2·22	1	224	4·94	0·563	0·107	0·139	0·317	11·40	40·9 ± 1·1	—	—	
,, 26	2·22	1	220	5·10	0·534	0·029	0·155	0·350	10·47	39·7 ± 0·9	—	—	
,, 27	2·22	1	190	4·98	0·528	0·024	0·155	0·349	10·60	39·8 ± 0·9	0·75	0·129	
,, 28	2·22	1	252	5·12	0·532	0·002	0·157	0·375	10·38	37·5 ± 0·8	—	—	63·4
,, 29	2·22	1	202	4·96	0·533	0·000	0·160	0·373	10·74	38·2 ± 0·8	—	—	
,, 30	—	1	181	4·60	0·276	0·069	0·049	0·158	6·00	58·0 ± 6·9	—	—	
Dec. 1	—	1	169	4·47	0·524	0·358	0·016	0·150	11·72	—	1·05	0·225	
,, 2	—	1	196	3·74	0·434	0·298	0·019	0·117	11·60	—	—	—	—
,, 3	—	—	152	3·58	0·262	0·157	0·008	0·097	7·31	—	—	—	—
,, 4	—	—	266	4·57	0·277	0·168	0·009	0·100	6·06	Normal day	0·78	0·133	—
,, 5	—	—	218	4·61	0·294	0·194	0·009	0·091	6·38	,,	—	—	—
,, 6	1·11	—	234	4·09	0·270	0·036	0·075	0·159	6·60	50·4 ± 3·2	—	—	55·6
,, 7	—	—	186	4·14	0·220	0·092	0·019	0·109	5·32	—	—	—	
,, 8	—	—	216	3·90	0·269	0·169	0·011	0·089	6·90	Normal day	—	—	—
,, 9	—	—	178	4·36	0·265	0·163	0·007	0·095	6·06	,,	0·95	0·149	—
,, 10	2·22	—	224	4·35	0·439	0·037	0·120	0·282	10·09	37·1 ± 1·1	—	—	58·6
,, 11	—	—	178	4·23	0·154	0·000	0·040	0·114	3·64	60·8 ± 9·6	0·81	0·128	
,, 12	—	—	152	3·16	0·115	0·032	0·012	0·071	3·64	—	—	—	—
,, 13	—	—	211	3·61	0·216	0·141	0·010	0·065	5·98	(?) Normal day	—	—	—
,, 14	—	—	208	3·43	0·207	0·131	0·007	0·069	6·02	,, ,,	—	—	—
,, 15	—	—	202	3·63	0·229	0·148	0·010	0·071	6·30	,, ,,	0·62	0·099	—
,, 16	—	—	224	3·72	0·234	0·148	0·010	0·076	6·29	,, ,,	—	—	—
,, 17	—	—	266	3·67	0·231	0·155	0·006	0·070	6·29	,, ,,	—	—	—
Mean normal output (av. 12 days)	—	—	219	3·99	0·245	0·152	0·009	0·084	6·13	—	—	—	—
Probable error	—	—	± 26	0·45	0·025	0·018	0·002	0·013	0·31	—	—	—	—
Mean normal output (av. 7 days, excluding Dec. 13 to 17)	—	—	217	4·26	0·260	0·157	0·009	0·094	6·10	—	—	—	—
Probable error	—	—	± 28	0·23	0·022	0·021	0·002	0·008	0·38	—	—	—	—

The values for the ratio $\frac{E}{E+NS}$ are based on the mean normal output for 7 normal days, excluding December 13 to 17, and on the mean output of neutral sulphur derived from 1 g. cystine (Exp. 12).

The dog's weight fell from 7·50 to 7·43 kilo. in 29 days.

Balance Sheet (29 days).

	T. N g.	T. S g.	T. N g.	T. S g.
Excreted in urine	121·80	9·660		
,, faeces	6·27	1·052		
			128·07	10·712
Given by mouth, food	126·73	9·889		
,, ,, cystine	1·28	2·933		
			128·01	12·822
		Balance	−0·06	+2·110

2·22 g. chlorobenzene were given by mouth on five consecutive days and 1 g. cystine on eleven consecutive days. On later dates single doses of 1·11 g. and 2·22 g. chlorobenzene were given for comparison with the preceding series. It will be noted that the output of neutral sulphur on the last five days is distinctly low. In determining the fate of the cystine these days should probably be excluded in arriving at a mean normal output, which is to be deducted from the neutral sulphur output on the cystine days. The administration of cystine was not continued sufficiently long to obtain a true value for the cystine effect.

Exp. 10. "Vixen," January 24 to February 18, 1922.

Table X. (Exp. 10.)

Diet as in Exp. 9.

Date 1922	Chloro-benzene by mouth g.	Urine: Vol. cc.	T. N g. N	T. S g. S	In. SO_4 g. S	Eth. SO_4 g. S	N. S g. S	T. S / T. N	E / E+NS	Faeces: T. N g. N	Faeces: T. S g. S	Percent. of dose chloro-benzene excreted with S
Jan. 24	—	194	3·50	0·203	0·104	0·012	0·087	5·80	Normal Day	—	—	—
,, 25	—	210	3·95	0·217	0·122	0·010	0·085	5·49	,,	—	—	—
,, 26	—	186	4·02	0·225	0·141	0·008	0·076	5·60	,,	—	—	—
,, 27	—	170	3·81	0·226	0·121	0·009	0·096	5·94	,,	1·14	0·188	—
,, 28	—	203	3·92	0·221	0·124	0·008	0·089	5·64	,,	—	—	—
,, 29	—	201	3·89	0·200	0·116	0·008	0·076	5·14	,,	—	—	—
,, 30	—	203	3·94	0·210	0·110	0·009	0·091	5·32	,,	—	—	—
,, 31	2·22	316	5·41	0·442	0·034	0·129	0·279	8·17	38·2 ± 0·9	—	—	
Feb. 1	2·22	256	4·91	0·358	0·000	0·077	0·281	7·29	25·8 ± 0·7	1·36	0·254	49·3
,, 2	2·22	220	5·21	0·329	0·000	0·067	0·262	6·31	24·7 ± 0·8	—	—	
,, 3	—	160	4·98	0·217	0·000	0·051	0·166	4·36	34·1 ± 2·0	—	—	
,, 4	—	130	4·75	0·108	0·000	0·028	0·080	2·27	—	—	—	
,, 5	—	237	4·34	0·196	0·103	0·004	0·089	4·51	—	0·86	0·158	—
,, 6	—	300	4·26	0·282	0·154	0·008	0·120	6·62	(?) Normal day	—	—	—
,, 7	—	260	4·23	0·275	0·156	0·005	0·114	6·50	,, ,,	—	—	—
,, 8	—	296	4·75	0·274	0·140	0·012	0·122	5·77	,, ,,	—	—	—
,, 9	—	232	4·56	0·283	0·162	0·008	0·113	6·21	,, ,,	1·11	0·162	—
,, 10	—	156	4·18	0·250	0·146	0·008	0·096	5·98	Normal day	—	—	—
,, 11	2·22	302	5·66	0·473	0·041	0·149	0·283	8·36	41·4 ± 0·9	—	—	
,, 12	—	160	5·15	0·158	—	0·041	0·117	3·07	50·0 ± 5·5	—	—	77·2
,, 13	—	243	5·02	0·327	0·179	0·014	0·134	6·50	—	—	—	
,, 14	—	202	4·68	0·308	0·178	0·018	0·112	6·58	—	—	—	
,, 15	—	194	4·45	0·260	0·166	0·010	0·084	5·84	Normal day	0·78	0·148	—
,, 16	—	193	4·17	0·260	0·177	0·009	0·074	6·24	,,	—	—	—
Mean normal output (av. 14 days)	—	214	4·12	0·242	0·139	0·009	0·094	5·86	—	—	—	—
Probable error	—	± 41	0·31	0·029	0·022	0·002	0·016	0·41	—	—	—	—
Mean normal output (av. 10 days, excluding Feb. 6 to 9)	—	191	3·98	0·227	0·133	0·009	0·085	5·70	—	—	—	—
Probable error	—	± 16	0·28	0·022	0·023	0·001	0·007	0·31	—	—	—	—
Feb. 17	—	265	5·89	1·660	1·275	0·008	0·377	28·20	—	0·64	0·125	—
,, 18	—	250	4·98	0·663	0·382	0·013	0·268	13·32	—	0·52	0·371	—

The values for the ratio $\frac{E}{E+NS}$ are based on the mean normal output for 10 normal days, excluding Feb. 6 to 9.

On Feb. 17 10 g. cystine were administered by mouth.

The dog's weight fell from 7·47 to 6·68 kilo. in 24 days.

Balance Sheet (Jan. 24 to Feb. 16, inclusive).

	T. N g.	T. S g.	T. N g.	T. S g.
Excreted in urine	107·74	6·302		
,, faeces	5·25	0·910		
			112·99	7·212
Given by mouth, food			104·88	8·184
		Balance	−8·11	+0·972

2·22 g. chlorobenzene were given by mouth on three consecutive days. It was not possible to continue such dosage alone for a longer period without the grave risk of upsetting the dog. On a later date 2·22 g. chlorobenzene were given as a single dose for comparison with the previous experiment. The neutral sulphur output from February 6 to 9 inclusive is distinctly greater than during the fore-period. It is doubtful whether these days should be taken into account in determining the mean normal output.

Exp. 11. "Vixen," April 6 to 21, 1922.

Table XI. (Exp. 11.)

Diet as in Exp. 9.

Date 1922	Chloro-benzene by mouth g.	Sodium sulphate by mouth g.	Urine Vol. cc.	T. N g. N	T. S g. S	In. SO_4 g. S	Eth. SO_4 g. S	N. S g. S	T. S / T. N	E / E+NS	Faeces T. N g. N	Faeces T. S g. S
April 6	—	—	176	3·81	0·209	0·129	0·005	0·075	5·49	Normal day	—	—
„ 7	—	—	216	4·03	0·253	0·151	0·007	0·095	6·28	„	—	—
„ 8	—	—	236	3·66	0·237	0·137	0·006	0·094	6·47	„	—	—
„ 9	—	—	250	4·27	0·248	0·155	0·008	0·085	5·80	„	—	—
„ 10	—	2·6	275	4·22	0·499	0·397	0·008	0·094	11·82	Sulphate day	1·27	0·201
„ 11	—	2·6	244	4·11	0·475	0·378	0·006	0·091	11·57	„	—	—
„ 12	—	2·6	220	4·33	0·509	0·397	0·007	0·105	11·76	„	—	—
„ 13	2·22	2·6	320	5·48	0·676	0·230	0·165	0·281	12·34	45·3 ± 1·2	—	—
„ 14	2·22	2·6	194	4·85	0·615	0·206	0·150	0·259	12·68	45·8 ± 1·3	0·95	0·182
„ 15	2·22	2·6	334	4·98	0·558	0·163	0·142	0·253	11·21	45·3 ± 1·4	—	—
„ 16	2·22	2·6	236	4·59	0·529	0·139	0·137	0·253	11·53	44·5 ± 1·4	—	—
„ 17	2·22	2·6	324	7·32	0·596	0·177	0·125	0·294	8·14	36·6 ± 1·0	—	—
„ 18	—	2·6	372	4·65	0·504	0·205	0·074	0·225	10·83	33·2 ± 1·5	0·83	0·125
„ 19	—	2·6	250	4·00	0·441	0·257	0·039	0·145	11·00	36·8 ± 3·9	—	—
„ 20	—	2·6	174	3·67	0·405	0·313	0·010	0·082	11·05	—	—	—
„ 21	—	—	215	4·02	0·252	0·165	0·009	0·078	6·26	Normal day	0·88	0·135
Mean normal output (av. 5 days)	—	—	218	3·96	0·240	0·147	0·007		6·06	—	—	—
Probable error	—	—	± 24	0·21	0·016	0·013	0·001	0·090	0·36	—	—	—
Mean output 3 sulphate days	—	—	246	4·22	0·494	0·391	0·007	0·009	11·72	—	—	—
Probable error	—	—	± 23	0·09	0·014	0·009	0·001		0·10	—	—	—

The dog's weight fell from 7·14 to 6·80 kilo. in 16 days.
The percentage of dose chlorobenzene excreted in combination with sulphur is 59·4.

Balance Sheet (16 days).

	T. N g.	T. S g.	T. N g.	T. S g.
Excreted in urine	71·99	7·006		
„ faeces	3·93	0·643		
	69·92	5·456	75·92	7·649
Given by mouth, food	—	2·841		
„ „ sodium sulphate			69·92	8·297
Balance			−6·00	+0·648

2·22 g. chlorobenzene were given by mouth on five consecutive days and 2·6 g. sodium sulphate (Na_2SO_4, $10H_2O$) on eleven consecutive days. Unfortunately the experiment had to be discontinued on the 16th day, because the glass catheter was accidentally broken in the dog's bladder.

THE FATE OF CYSTINE.

Exp. 12. "Vixen," January 30 to February 13, 1923 (Table XII).

1 g. cystine was administered by mouth on seven consecutive days.

In Exp. 12 the administration of 1 g. cystine (equivalent to 0·266 g. S) is followed by the additional elimination of 0·035 g. of neutral sulphur or 13 % of the dose, of 0·196 g. S as sulphate or 74 % of the dose. This leaves 0·035 g. S or 13 % of the dose unaccounted for.

Table XII. (Exp. 12.)

Diet as in Table IX. Cystine 1 g. daily by mouth February 3 to 9.

Date 1923	Urine: Vol. cc.	T. N g. N	T. S g. S	In. SO_4 g. S	Eth. SO_4 g. S	N. S g. S	T. S / T. N	Faeces: T. N g. N	T. S g. S
Jan. 30	292	5·02	0·273	0·180	0·011	0·082	5·43	—	—
,, 31	189	4·66	0·288	0·192	0·011	0·085	6·18	—	—
Feb. 1	242	4·92	0·289	0·202	0·009	0·078	5·87	—	—
,, 2	218	4·75	0·278	0·192	0·010	0·076	5·85	—	—
,, 3	230	4·95	0·496	0·387	0·007	0·102	10·03	0·61	0·114
,, 4	238	4·36	0·484	0·364	0·008	0·112	11·10	—	—
,, 5	260	4·90	0·530	0·392	0·011	0·127	10·82	0·52	0·115
,, 6	200	4·83	0·514	0·382	0·010	0·122	10·64	—	—
,, 7	330	5·03	0·519	0·390	0·011	0·118	10·31	—	—
,, 8	220	4·83	0·526	0·393	0·010	0·123	10·90	—	—
,, 9	241	5·01	0·523	0·395	0·007	0·121	10·44	—	—
,, 10	230	4·94	0·300	0·207	0·011	0·082	6·07	0·84	0·185
,, 11	248	4·84	0·305	0·194	0·011	0·100	6·30	—	—
,, 12	218	4·83	0·268	0·179	0·006	0·083	5·54	—	—
,, 13	281	4·55	0·256	0·164	0·012	0·080	5·62	—	—
Mean normal output (av. 8 days)	240	4·81	0·282	0·189	0·010	0·083	5·86	—	—
Probable error	± 32	0·14	0·015	0·013	0·002	0·007	0·29	—	—
Mean output 7 cystine days	246	4·84	0·513	0·386	0·009	0·118	10·61	—	—
Probable error	± 38	0·21	0·016	0·010	0·002	0·007	0·34	—	—

The dog's weight fell from 8·14 to 7·94 kilo. in 15 days.

Balance Sheet (15 days).

	T. N g.	T. S g.	T. N g.	T. S g.
Excreted in urine	72·42	5·849		
,, faeces	1·97	0·414		
			74·39	6·263
Given by mouth, food	65·55	5·115		
,, ,, cystine	0·82	1·867		
			66·37	6·982
		Balance	−8·02	+0·719

In Exp. 9 the mean normal output of neutral sulphur over the control periods, excluding the last five days is 0·094 g. S. If to this figure is added the neutral sulphur output due to 1 g. cystine (Exp. 12) the value obtained is 0·129 g. S, a value almost identical with the output of neutral sulphur on the two days previous to the administration of the chlorobenzene. It appears therefore fair to apply the conclusions arrived at in Exp. 12 to Exp. 9.

It is safer to assume that all the sulphate on the chlorobenzene days is derived from the administered cystine. If it is assumed that part of this fraction is derived from protein, the figure for the utilisation of the cystine in forming mercapturic acid must be correspondingly increased. The output of sulphate on the five chlorobenzene days is given in Table XIII.

Table XIII.

(Derived from Table IX.)

Date 1921	In. SO_4 g. S	Eth. SO_4 g. S	Total SO_4 g. S	Difference from 0·196 g. S, figure for SO_4 derived from cystine given alone g. S
Nov. 25	0·107	0·139	0·246	—
,, 26	0·029	0·155	0·184	0·012
,, 27	0·024	0·155	0·179	0·017
,, 28	0·002	0·157	0·159	0·037
,, 29	0·000	0·160	0·160	0·036

On the first day the output of sulphate is much greater than the average rise in sulphate after 1 g. cystine, given alone. In this experiment as in all other experiments it is clear that at first there is a certain amount of sulphate in the cell available for the detoxication of the chlorobenzene. On the following days the sulphate figure gradually falls, until on the last two days there is a difference of 0·036 g. S, a figure indicating that 14 % of the dose has been excreted as mercapturic acid. This is the minimum figure. If all twelve normal days are taken for the calculation of the mean normal output of neutral sulphur, this figure becomes 0·046 g. S or 17 % of the dose. It is also seen that all the sulphate appears ultimately as ethereal sulphate. It may be stated therefore that on the last two days 14 to 17 % of the cystine given appears as mercapturic acid, 57 to 60 % as ethereal sulphate. The higher sulphate output on the other days may quite well be due to traces of sulphate in the tissues derived from other sources.

On November 30, the day following the last chlorobenzene day, the output of sulphur is remarkable, in that it is reduced to the very small figure 0·276 g. S. This would suggest at once that there had been some error in the administration of cystine or in the absorption of cystine from the gut. No such error is known to have occurred. The figure is not an impossible one. It has been observed that the average output of sulphur after 1 g. cystine is 0·231 g. S. If this value is deducted from the sulphur output on the day in question, there still remains a balance for the sulphur derived from the protein. There is always a marked retention of sulphur after the administration of chlorobenzene, a fact well exemplified in Exp. 10, where there is no question of error. If it is assumed that there has been no error, this day affords clear proof of the direct conversion of cystine to mercapturic acid or other neutral sulphur compound. Experimental confirmation however is obviously required before the analyses for this day can be accepted.

The fate of sodium sulphate.

This question is not so difficult as the last, as the administered sulphate can only appear as inorganic or as ethereal sulphate.

In Exp. 11 the average output of total sulphate on the three sulphate days is 0·398 g. S and on the five normal days is 0·154 g. S. This leaves a balance due to 2·6 g. sodium sulphate of 0·244 g. S.

The outputs of inorganic sulphate and of ethereal sulphate on the chlorobenzene days are given in Table XIV.

Table XIV.

(Derived from Table XI.)

Date 1922	Eth. SO_4 g. S	In. SO_4 g. S	Difference from 0·244 g. S g. S	% dose sodium sulphate excreted as ethereal SO_4
April 13	0·165	0·230	0·014	5
„ 14	0·150	0·206	0·038	15
„ 15	0·142	0·163	0·081	31
„ 16	0·137	0·139	0·105	40
„ 17	0·125	0·177	0·067	26

In making calculations it is safer to assume that all the inorganic sulphate excreted after the chlorobenzene is derived from the administered sulphate and the ethereal sulphate is mostly formed from protein. On this assumption the percentages shown in the last column of the table are reached. There is no evidence that on the first day any of the administered compound has been utilised in detoxicating the chlorobenzene. On the remaining days from 15 to 40 % of the dose appears as ethereal sulphate.

The calculations may be made in a different manner. If the actual excess outputs of ethereal sulphate and of neutral sulphur over the experimental days are compared, Table XV is obtained.

Table XV.

	Cystine experiment		Control experiment		Sulphate experiment	
Day	Eth. SO_4 g. S	N. S g. S	Eth. SO_4 g. S	N. S g. S	Eth. SO_4 g. S	N. S g. S
1	0·130	0·188	0·120	0·194	0·158	0·191
2	0·146	0·221	0·068	0·196	0·143	0·169
3	0·146	0·220	0·058	0·177	0·135	0·163
4	0·148	0·246	—	—	0·130	0·163
5	0·151	0·244	—	—	0·118	0·204

In constructing this table the figures taken for the mean normal output of neutral sulphur are such as would reduce the effect of the cystine in forming mercapturic acid to the lowest possible value. Any other combination would increase the value of the cystine effect.

There is but little difference between the figures for the first day of each experiment. Apparently sufficient sulphate was present in the tissues to obscure the issue. This bears out the failure to show any direct synthesis in the earlier experiments (Nos. 4, 5). On the remaining days however the effect of these added substances is quite well marked. There is much more ethereal sulphate in the cystine and sulphate experiments as compared with the output in the control experiment. In addition to this there is a distinct increase in the output of neutral sulphur in the cystine experiment. If the first day of each series is neglected and the mean values for the remaining days are taken, Table XVI is obtained.

Table XVI.

	Cystine experiment g. S	Control experiment g. S	Sulphate experiment g. S
Excess Eth. SO_4	0·148	0·063	0·131
Excess Neut. S	0·233	0·186	0·175
Sum	0·381	0·249	0·306
$\frac{E}{E+NS}$	38·8	25·3	42·8
% chlorobenzene excreted with S	60·2	39·4	48·4
Increase of Eth. SO_4 over control	0·085	—	0·068
Expressed as % of dose S given	32·0	—	26·1
Increase of Neut. S over control	0·047	—	—
Expressed as % of dose S given	17·7	—	—

It must be noted in the above Table XVI that the figures are based on the averages of four days (two days in the case of the control experiment) and therefore differ from those given in the tables of the actual experiments.

These figures are similar to those obtained by the first method of calculation, except for the amount of ethereal sulphate, derived from the cystine. In the first method it is assumed that all the ethereal sulphate is derived from the cystine, in the second it is assumed that some is derived from protein. The first method of calculation is probably the more correct. In the control experiment more protein was being catabolised, as evidenced by the greater output of nitrogen. The cystine and to a lesser degree the sulphate partly prevented this tissue waste.

However these results may be interpreted, one thing is clearly defined, the comparative constancy in the relative outputs of ethereal sulphate and neutral sulphur. The mean value for the $\frac{E}{E+NS}$ ratio in the cystine experiment is 38·8, for the sulphate experiment is 42·8. If anything, the output of ethereal sulphate (relative to that of neutral sulphur) is increased by the addition of sulphate to the diet and the output of neutral sulphur (relative to that of ethereal sulphate) is increased by the addition of cystine to the diet, though with cystine there is an absolute increase of both. There has been some mass-action effect on the velocities of the two synthetic reactions but the effect is small in comparison with the doses given.

It is interesting to note that in the control experiment there is a marked falling off in the output of ethereal sulphate on the second and third days, though the output of neutral sulphur does not change. Exp. 2 shows the same phenomenon. This would seem to indicate that the production of sulphate from protein is slower than the production of sulphate from cystine, so that the ratio becomes altered. The alteration in the ratio could also be attributed to the increase in tissue breakdown (shown by the T. S/T. N ratio and the output of nitrogen) and the production of a neutral sulphur complex, not mercapturic acid.

Discussion.

Sulphur and nitrogen balance.

It will be observed on looking through the tables that the nitrogen balance in the majority of the experiments is not very far out but that in general there is a marked positive balance for sulphur. This cannot be a question of lag in the sulphur excretion or entirely due to sulphur retention by the dog as the retention bears no relation to the state of nutrition of the animal. Wolf and Österberg [1911] and Lewis [1916] have discussed the question of sulphur retention in relation to periods of protein feeding after starvation, but although in some of the earlier experiments the state of nutrition of the animal improved during the course of the experiment, there was no alteration in the diet and the improvement was slight. The positive sulphur balance is much more

likely to be due to some definite experimental error or other cause. The difficulty of obtaining satisfactory sulphur analyses for the biscuit has already been discussed but the error is more likely to be in the direction of using too low a value for the sulphur of the biscuit than too high. There is a considerable loss of sulphur by the hair. The dogs were losing hair throughout the experiments and this loss was not estimated, as the relation between the loss of hair and the growth of hair was not known. Rough calculations of the quantity of hair on each dog gave the following figures: Phoena, 100 g.; Diana, 80 g.; Vixen and Sophia 75 g. Hair contains about 5 % of sulphur, so that there was 3·5 to 5 g. S in the hair of each dog. Assuming growth to be regular (which no doubt is not true) and the whole coat to be changed every twelve months, the daily consumption of sulphur in forming hair would be 10 to 15 milligrams, an appreciable percentage of the sulphur unaccounted for. There is also a loss of sulphur in estimating the sulphur of the faeces. Faeces are rich in sulphur and are not always easily collected from the metabolism cages. The loss of sulphur on the total would be relatively greater than that of the nitrogen. There must also be some loss of sulphur from the dogs in volatile compounds, though not necessarily of any considerable weight. Volatile compounds were particularly noticeable in the dog used in Exp. 10 after the dose of 10 g. cystine.

The output of neutral sulphur in relation to the diet.

The rise in the neutral sulphur of the urine (Exp. 6) on increasing the protein of the diet is a very marked feature. The dog was probably receiving more protein than it could digest, as it lost appetite. The large increase in the output of the neutral sulphur was almost certainly associated with some increased bacterial activity in the intestine, as the ethereal sulphate was somewhat raised.

The elimination of sulphur after cystine.

The increased output of sulphur following the administration of cystine was not observed for longer than 48 hours. This was the maximum period of lag in sulphur excretion. This lag has been considered in arriving at the mean output on normal days. As recorded by Goldmann in dogs [1885], Wohlgemuth in rabbits [1903], Blum in dogs [1904] and confirmed by Schmidt and Clark in dogs [1922] and by Sherwin and Rose in rabbits [1923] there may be a considerable rise of neutral sulphur after cystine, presumably in part from products of bacterial activity in the gut, though Sherwin and Rose attribute some at any rate of the "extra" neutral sulphur to incomplete oxidation of the cystine after absorption and state that apparently slow absorption facilitates oxidation.

On no occasion was the dose of cystine fully oxidised, though Rothera in man [1905] and Lewis and Root in rabbits [1922] were able to demonstrate the complete oxidation of this substance. Schmidt and Clark [1922] found

cystine in the urine of a dog which had received 7·5 g. by mouth. They attributed this to unusually rapid absorption. Perhaps the animal was an intermittent cystinuric [Garrod, 1923]. No cystine could be demonstrated by Gaskell's method [1907] in the urine of the dog used in Exp. 10 after a dose of 10 g. cystine, though the rise in neutral sulphur corresponded to 1·77 g. In this instance there was marked bacterial activity in the intestine, demonstrated by the evolution of much hydrogen sulphide from the anal canal.

The effect of chloro- and bromo-benzene on metabolism.

The effect of these substances on sulphur metabolism is best seen in the $\frac{T.S}{T.N}$ and $\frac{E}{E+NS}$ ratios. These and the phenol ratio are shown in Table XVII.

Table XVII.

Exp.	Dog	Dose g.	Day after dose	$\frac{T.S}{T.N}$	$\frac{E}{E+NS}$	Phenol ratio
Chlorobenzene:						
4	Phoena	Control period		5·28±0·27		
		0·55	1	5·51	46·5±4·0	40·9±2·9
		3·33	1	8·19	38·3±1·0	34·1±1·0
		—	2	4·10	46·9±3·0	38·2±2·2
		—	3	3·40	30·2±4·8	32·8±7·4
6	Phoena	Control period		5·47±0·30		
		3·33	1	5·92	—	38·7±0·8
		—	2	4·93	—	36·4±1·7
8	Phoena	Control period		4·88±0·25		
		0·37	1	5·00	36·6±4·2	27·9±4·5
		0·28	1	5·38	31·7±3·7	12·7±7·0
		2·22	1	7·18	40·2±1·0	31·4±1·1
		—	2	3·54	40·5±3·0	40·2±2·4
		0·55	1	5·83	37·3±2·5	29·6±3·1
		2·22	1	7·60	38·8±1·0	31·5±1·1
		—	2	3·90	35·2±2·3	33·1±2·7
9	Vixen	Control period		6·10±0·38		
		1·11	1	6·60	50·4±3·2	—
		2·22	1	10·09	37·1±1·1	—
		—	2	3·64	60·8±9·6	—
10	Vixen	Control period		5·70±0·31		
		2·22	1	8·36	41·4±0·9	—
		—	2	3·07	50·0±5·5	—
Repeated doses of chlorobenzene:						
10	Vixen	Control period		5·70±0·31		
		2·22	1	8·17	38·2±0·9	—
		2·22	1	7·29	25·8±0·7	—
		2·22	1	6·31	24·7±0·8	—
		—	2	4·36	34·1±2·0	—
		—	3	2·27	—	—
Chlorobenzene with sodium sulphate:						
4	Phoena	Control period		—		
		3·33	1	12·22	40·9±1·0	38·6± 1·0
		—	2	10·02	44·3±2·2	41·7± 2·3
		—	3	12·00	42·2±4·5	30·4±11·1
5	Phoena	Control period		—		
		3·33	1	13·03	42·9±1·0	34·1± 0·9
		—	2	10·18	36·8±1·7	47·9± 1·3
		—	3	4·29	41·0±5·1	45·2± 3·3

Table XVII (*continued*).

Exp.	Dog	Dose g.	Day after dose	T. S / T. N	E / E+NS	Phenol ratio
Repeated doses of chlorobenzene with sodium sulphate:						
11	Vixen	Control period		11·72±0·10		
		2·22	1	12·34	45·3±1·2	—
		2·22	1	12·68	45·8±1·3	—
		2·22	1	11·21	45·3±1·4	—
		2·22	1	11·53	44·5±1·4	—
		2·22	1	8·14	36·6±1·0	—
		—	2	10·83	33·2±1·5	—
		—	3	11·00	36·8±3·9	—
Chlorobenzene with cystine:						
5	Phoena	Control period		—		
		3·33	1	13·14	—	42·0±0·7
		—	2	5·00	—	43·0±1·6
		3·33	1	13·29	—	42·7±0·7
		—	2	11·03	—	40·2±1·6
		—	3	6·04	—	38·8±5·2
Repeated doses of chlorobenzene with cystine:						
9	Vixen	Control period		(11·14)		
		2·22	1	11·40	40·9±1·1	—
		2·22	1	10·47	39·7±0·9	—
		2·22	1	10·60	39·8±0·9	—
		2·22	1	10·38	37·5±0·8	—
		2·22	1	10·74	38·2±0·8	—
		—	2	6·00	58·0±6·9	—
Bromobenzene:						
7	Sophia	Control period		4·41±0·53		
		0·5	1	4·28	38·1±3·4	—
		3·0	1	7·24	37·0±0·9	—
		—	2	4·00	32·2±1·9	—
		0·75	1	5·72	28·4±1·7	—
		0·37	1	5·10	25·3±3·0	—
Repeated doses of bromobenzene:						
2	Judy	Control period		(5·28)		
		3·0	1	7·60	(45·7)	—
		5·0	1	7·14	(30·9)	—
		5·0	1	6·34	(18·2)	—
		2·0	1	5·90	(24·0)	—

The probable error could not be calculated for the figures in brackets, as the experiment was not continued long enough to determine a proper average for the mean normal output of the various sulphur fractions of the urine.

The analytical error has not been taken into account in calculating the probable error, so that the latter is somewhat greater. It must however be remembered that a constant analytical error would have no effect on the $\frac{E}{E+NS}$ ratio. It is obvious that to obtain a true value for this ratio a very large number of observations ought to be made. This is especially true for the smaller doses of bromo- or chloro-benzene.

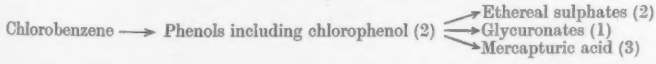
The figures are however sufficiently striking to warrant attention being drawn to them. Where the variation is greatest, the probable error is usually greatest. The phenol ratio also shows some constancy except in Exp. 6. In this experiment the estimations of phenol were not carried out on the fresh urine and perhaps this series ought to be omitted. It is unfortunate that the

estimations of the phenols were not done throughout. They were discontinued because the results did not appear to have any meaning. The significance of the figures was only discovered at a very much later date.

The constancy of the $\frac{E}{E+NS}$ ratio is evidence for the balance between reactions taking place in the cell. The rate of production of ethereal sulphate to that of mercapturic acid bears the ratio of roughly 4 to 6. The assumption is made that the elimination of the two final products takes place with equal ease. In the first paper of this series [Hele, 1924], in order to account for the conflicting evidence of other workers on the synthesis of ethereal sulphate, the assumption was made that the rates of elimination of sulphate, sulphite and cystine by the kidney are different. This hardly amounts to an assumption as there is indirect evidence to substantiate it. On the other hand the assumption that mercapturic acid and ethereal sulphate are eliminated with equal ease has no experimental justification. If however the permeability of the kidney to these substances is markedly different, one would expect the $\frac{E}{E+NS}$ ratio to alter until the accumulation of end-products has established an equilibrium by the adjustment of the velocities of the syntheses to the rates of elimination. How rapidly such an adjustment would take place is not clear. The equilibrium should be attained more rapidly with large doses than with small. The work already done shows no marked difference between small and large doses. Perhaps by shortening the time interval differences would appear. Whatever causes however contribute to the results, the fact remains that under the experimental conditions a constant mixture is excreted by the kidney.

The phenol is roughly again four parts of free to six parts of total phenol. Here the story is much more complex. Unfortunately Folin's tungstate reagent is a group reagent, reacting with different degrees of intensity with different phenols (as well as with other substances) and it is not known how many phenols may arise from chlorobenzene. It is possible however that the products of oxidation may be distributed between the several fractions according to some definite proportion, so as to yield a constant relationship between free and conjugated phenols. Conjugated phenols include the sulphates and the glycuronates and there may be others.

The several reactions may be summarised thus:



The numbers in brackets indicate the molecular proportion of the several fractions excreted, that would satisfy the numerical relations given above. It is not known how much, if any, glycuronic acid is excreted in direct combination with phenols after chlorobenzene. Estimations of glycuronic acid would not be sufficient to determine this point, as mercapturic acid itself is excreted in combination with glycuronic acid [Baumann, 1883]. Isolation of

the products will alone give the correct answer. If such glycuronic acids are absent or present in very small amounts, the proportions in the above scheme must be altered. This question will be discussed further in the third paper of this series. It is interesting to note that on such a scheme the theoretical yield of mercapturic acid is only 37 %. This is in general agreement with the increased output of neutral sulphur after chlorobenzene and helps to explain the small yield of mercapturic acid (equivalent to 20–30 % of the dose of chlorobenzene) obtained from urine even under the most favourable conditions. The correspondence may however be accidental.

There is accumulative evidence that the liver is the chief seat of the sulphate and glycuronic acid syntheses. The subject has been discussed in the first paper of this series [Hele, 1924]. There is also evidence that the liver is the organ mainly responsible for the mercapturic acid synthesis. Zeller and Straczewski [1914] could not obtain more than 0·2 g. mercapturic acid from the urine of a dog receiving bromobenzene (28 g.) on a protein-free diet, though the rise in neutral sulphur indicated the presence of 6·9 g. of the acid. In another experiment they failed to demonstrate the presence of any mercapturic acid. They attributed the rise in neutral sulphur to compounds other than mercapturic acid and suggested that in the breakdown of tissue protein by bromobenzene on a protein-free diet no cystine is liberated and it is only when protein is included in the diet that mercapturic acid is formed. Thomas and Straczewski [1919] added *p*-bromophenylmercapturic acid to normal urine and were able to demonstrate its presence, when in concentration of only 0·2 g. in 500 cc. They administered bromobenzene *sub cutem* to dogs on a protein-rich diet. To one dog they gave 22·5 g. and to another 15·5 g. bromobenzene and only obtained traces of mercapturic acid from the urine. Kapfhammer [1921] administered in eight days 12 g. bromobenzene by mouth to a dog on a protein-free diet and failed to obtain any mercapturic acid from the urine. He then administered in four days 7 g. bromobenzene to the same dog on a protein-free diet and at the same time injected 29 g. of cystine *sub cutem*. He obtained 2·86 g. of impure mercapturic acid. These authors conclude that mercapturic acid is synthesised in the liver and from food protein only. Under these circumstances therefore one would expect to be able to alter the ratios of the syntheses in the liver by the simultaneous administration of cystine or of sodium sulphate with the bromo- or chlorobenzene by the mouth. Rhode [1922] has shown that the administration of cystine subcutaneously cuts down the output of ethereal sulphate, produced by bromobenzene given by the mouth. Such a method of administration apparently increases the velocity of the synthesis of mercapturic acid over that of ethereal sulphate. Unfortunately he carried out no estimations of neutral sulphur, no doubt because the cystine apart from the bromobenzene would have affected his results. Muldoon, Shiple and Sherwin [1923, 1, 2] administered bromobenzene with sulphate and with cystine by mouth to a dog. The sulphate was not converted to ethereal

sulphate though the cystine was in part converted to (?) mercapturic acid. The administration however both of the sulphate and of the cystine was only continued for two days. This may account for the failure in the utilisation of the sulphate. A full account of their work has not yet appeared.

In the experiments described in this paper the simultaneous oral administration of sulphate or of cystine had practically no effect on the relative output of ethereal sulphate and of neutral sulphur, though the total effect was considerable. Sodium sulphate was used in the synthesis of ethereal sulphate and thus there was more protein available for the formation of mercapturic acid. Cystine appeared largely as ethereal sulphate, though a small percentage appeared as mercapturic acid. The reactions may therefore be written:

$$\text{Chlorobenzene} \rightarrow \text{Chlorophenol} \begin{cases} + \text{Cystine} \rightarrow \text{Mercapturic acid} \\ + \text{Sulphate} \rightarrow \text{Ethereal sulphate} \end{cases}$$

Under these experimental conditions oxidation of the cystine precedes the other reactions or the rate of oxidation is influenced by the other reactions.

The constancy of the $\frac{E}{E+NS}$ ratio presents one difficulty to the theory of Thomas, Zeller and Straczewski. It is noticeable in the table of ratios, that the bromobenzene or chlorobenzene produces an immediate rise in the $\frac{T.S}{T.N}$ ratio with afterwards a compensatory fall. Larger doses produce a change of nearly 100 %. It is difficult to conceive that this output of sulphur only comes from food protein, as there is no known mechanism of storing amino-acids in quantity save as protoplasm. The more natural interpretation is that the toxic substances act directly on the cell with the liberation of sulphur complexes and that the later output of nitrogen represents the adjustment necessary to restore the normal cell-content of amino-acids. If this process involved the production of another sulphur compound of bromo- or chlorobenzene, an alteration in the $\frac{E}{E+NS}$ ratio would be expected, just as there is an alteration in the $\frac{T.S}{T.N}$ ratio. Yet no such alteration takes place, when the dose of bromo- or chlorobenzene is not repeated.

It is true that on repeating the doses of these substances there is a marked fall in the $\frac{E}{E+NS}$ ratio. This fall is always associated with the onset of signs of poisoning in the dog (fever, lassitude, loss of appetite and vomiting). If the sulphur analyses given in the paper by Marriott and Wolf [1907] on bromobenzene poisoning are examined, the same fact is made manifest. The control periods are hardly long enough to yield a true mean normal output, but so far as the analyses can show, the $\frac{E}{E+NS}$ ratio lies between 37·5 and 44·8, except in one experiment. In this experiment the dog was starved and given bromobenzene repeatedly. The ratio fell from 35·1 to 5 and then the dog died.

This fall would be readily explicable on the theory of Thomas, Zeller and

Straczewski but could be explained equally well on other lines. It has been noted that on the first day of such continued administration of chlorobenzene, cystine or sulphate added to the diet has no apparent effect. There is presumably enough sulphate in the cell to yield all the ethereal sulphate formed. On the second and later days, when sulphate or cystine is not given, the sulphate may be deficient in the cell and the oxidation of the protein cystine may not proceed so rapidly as its removal by the chlorobenzene to form mercapturic acid resulting in an alteration of the ratio. All the experiments in this paper bear out such a theory. However until a mercapturic acid has been isolated in sufficient quantity from the urine of dogs on a protein-free diet, it is clear that the theory of Thomas, Zeller and Straczewski must be accepted and another explanation sought for the constancy of the $\frac{E}{E+NS}$ ratio.

There is also a marked fall in the $\frac{T.S}{T.N}$ ratio after the prolonged administration of guaiacol [Hele, 1924]. This would suggest that even guaiacol has some slight toxic action on sulphur metabolism, too small to cause an appreciable rise of neutral sulphur during its administration, the effect being accumulative and only manifest after the dosage has been stopped.

It is frequently stated that species other than the dog are unable to synthesise mercapturic acid. Certainly it does not follow that the requisite enzymes are present in all species or, if present, are present in the same concentrations, but, apart from this, account is not always taken of the dosage given and the difficulty of separating mercapturic acid in low concentrations. For instance Mester [1890] administered doses of 4 g. and 6 g. bromobenzene respectively to two men and although he obtained a small rise in neutral sulphur, he was unable to demonstrate the presence of a mercapturic acid. It is true that in the experiments recorded in this paper on the dog, small doses yielded a value for the $\frac{E}{E+NS}$ ratio similar to that yielded by large doses. The percentage yield of mercapturic acid was presumably the same in the two cases, but, at the concentration in the urine after small doses, it would be difficult to obtain any definite evidence of the presence of a mercapturic acid. A 6 g. dose for a man would yield no higher concentration of mercapturic acid in the urine than a 0·5 g. dose in the dog. Mester's work is evidence as much for the synthesis of a mercapturic acid by man as the reverse.

Summary.

1. Chlorobenzene administered by the mouth has an approximately constant quantitative effect on the sulphur metabolism of the dog. The "extra" ethereal sulphate bears a constant proportion (about 2 to 3) to the "extra" neutral sulphur. In this paper the relation is expressed as a ratio $\frac{\text{"Extra" Eth. } SO_4 \times 100}{\text{"Extra" Eth. } SO_4 + \text{"Extra" Neut. S}}$ or $\frac{E}{E+NS}$.

2. This ratio does not vary within the experimental error with the dose or with the dog.

3. Sulphur is eliminated very much more rapidly than the nitrogen after chlorobenzene. The ratio $\frac{\text{Total sulphur} \times 100}{\text{Total nitrogen}}$ or $\frac{\text{T. S}}{\text{T. N}}$ may be increased nearly 100 % with a compensatory fall afterwards. The increased elimination of sulphur must be derived in part from the tissues. The alteration in the $\frac{\text{T. S}}{\text{T. N}}$ ratio is not reflected in the $\frac{\text{E}}{\text{E}+\text{NS}}$ ratio.

4. The simultaneous oral administration of cystine or of sodium sulphate does not appreciably alter the $\frac{\text{E}}{\text{E}+\text{NS}}$ ratio, though when the dose of chlorobenzene is repeated both compounds take part in the syntheses. (After the first chlorobenzene day cystine 14 to 17 % as (?) mercapturic acid and 57 to 60 % as ethereal sulphate: sodium sulphate 26 to 40 % as ethereal sulphate.)

5. The $\frac{\text{E}}{\text{E}+\text{NS}}$ ratio may be altered by repeated doses of chlorobenzene, if no cystine or sulphate is given at the same time. A low ratio is associated with the onset of toxic signs in the dog.

6. It is suggested that the constancy of the $\frac{\text{E}}{\text{E}+\text{NS}}$ ratio is proof under the experimental conditions of a balance between three series of reactions in the cell, the synthesis of mercapturic acid, the synthesis of ethereal sulphate and the oxidation of cystine to sulphate, the velocities of the several reactions being dependent on the concentrations of the catalysts and the effective masses of the reacting compounds at the seat of the syntheses. An explanation is advanced for the fall in the value of the ratio on repeating the dose of chlorobenzene.

7. The constancy of the $\frac{\text{E}}{\text{E}+\text{NS}}$ ratio seems to be antagonistic to the theory of Thomas, Zeller and Straczewski, that there are two neutral sulphur compounds of halogen substituted benzenes (bromobenzene), mercapturic acid formed in the liver from food protein and an unknown compound formed from tissue protein. If these compounds were formed at the same velocity, the $\frac{\text{E}}{\text{E}+\text{NS}}$ ratio would not then alter, whatever alterations the $\frac{\text{T. S}}{\text{T. N}}$ ratio underwent. There seems no reason however why the velocities should be identical. On the other hand the fall in the $\frac{\text{E}}{\text{E}+\text{NS}}$ ratio on repeating the dose of chlorobenzene would be readily explained on the theory of Thomas, Zeller and Straczewski.

8. As chlorobenzene as well as guaiacol unites directly with preformed sulphate there is no necessity to regard mercapturic acid as an intermediary stage in the formation of ethereal sulphate.

9. The ratio of "extra" free phenol to "extra" total phenol ($\frac{\text{Free phenol} \times 100}{\text{Total phenol}}$ or the Phenol Ratio) also appears to be constant. The figures are however less satisfactory.

10. There is no increase of free phenols in the faeces after chlorobenzene. In accordance with the observations of Folin and Denis no conjugated phenols occur in the faeces.

11. The action of bromobenzene appears to be similar to that of chlorobenzene.

12. In each experiment there was a marked positive balance for sulphur, even when the nitrogen intake and output corresponded closely. It is suggested however that this positive balance may be due to experimental error, loss of sulphur in the faeces, loss of sulphur in volatile compounds and to the growth of hair.

13. The oral administration of cystine always produced a small rise in the output of neutral sulphur. It is not clear whether this was due to products of intestinal decomposition or to failure in oxidation after absorption.

14. A rise in the protein intake may give a considerable rise in the output of neutral sulphur, possibly intestinal in origin.

15. Guaiacol appears to have some slight toxic action on sulphur metabolism, as, although there is no apparent rise in neutral sulphur during its administration, there is a marked fall in the $\frac{\text{T. S}}{\text{T. N}}$ ratio when the administration is stopped.

16. Too little account has often been taken of the dosage, when bromobenzene has been administered to individuals of different species. Thus Mester's work is as much in favour of the occurrence of the mercapturic acid synthesis in man as the reverse, though it is generally stated on the evidence of his work that man does not synthesise mercapturic acid.

My thanks are due to Prof. F. G. Hopkins for his interest displayed in this research and to my wife for valuable assistance in carrying out the experiments.

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LXXX. FURTHER OBSERVATIONS ON CERTAIN REDUCING AND OXIDISING REACTIONS IN MILK.

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(*Received April 16th, 1924.*)

In a recent paper dealing with certain reducing and oxidising reactions in milk [Haas and Hill, 1923] it was suggested that the substance there described as itate might possibly be identical with milk peroxidase. Subsequent experiments have shown, however, that this is not the case, and that the two substances are distinct.

With the object of obtaining some further information regarding the nature of itate, experiments were made upon the nitrite oxidising power of whole milk and of whey. It was then found, as the following experiment shows, that when milk is coagulated by rennet itate remains in the whey[1].

Exp. 1. Into a series of wide mouthed bottles fitted with corks and containing 75 cc. of milk and 1 cc. of 10 % acetic aldehyde, were placed 4, 5, 6, 7, 8, 9, 9·5, 10, 10·5, 11, 11·5, 12 and 13 cc. respectively of 0·182 % sodium nitrite; an exactly similar series of bottles was set up containing whey[2] in place of milk. These bottles were rotated in a thermostat at 45°, and the time in minutes required for the oxidation of the nitrite in each case was determined by testing at short intervals with Griess-Ilosvay reagent. The results so obtained are incorporated in the curves of Fig. 1.

It should be pointed out that employing the above method of experiment it is only possible to obtain approximately, to within five minutes, the time taken to oxidise a given quantity of nitrite. For this reason all the points do not fall onto a smooth curve. A comparison of the two curves, however, reveals the fact that the rate of oxidation of nitrite is slower in whey than in milk, and also that the total amount oxidised by the whey is less than that oxidised by the milk.

[1] One of us (P.H.) is indebted to Dr S. S. Zilva of the Lister Institute for having drawn his attention to this fact. The statement made in the earlier paper to the effect that "itate is precipitated with the proteins when milk is coagulated" referred to precipitation by saturation with ammonium sulphate, not rennet.

[2] The whey was prepared by coagulating milk with Benger's Essence of Rennet at 37° using 2 cc. of rennet for every 100 cc. of milk, the curds being filtered off through butter muslin. This rennet was shown by experiment to be free from nitrite and to be incapable of oxidising nitrite in the presence of acetic aldehyde.

The non-identity of itate and peroxidase.

It has been known for some time that when milk is coagulated by rennet, the peroxidase remains in the whey and the fact above established, that itate likewise remains in the whey, appeared to lend additional support to the possibility of the two substances being identical. On further fractionating the whey however, by half-saturation with ammonium sulphate, it was found that the precipitate (1), so obtained, when redissolved in water, was able to oxidise nitrite in the presence of acetic aldehyde, but on completely saturating the filtrate from precipitate (1), the new precipitate (2) when redissolved in

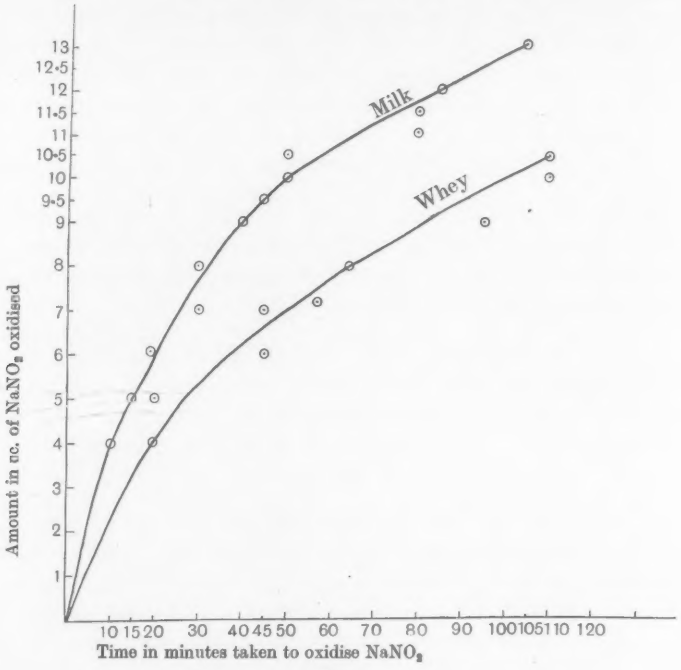

Fig. 1.

water was unable to do so. The solutions of both precipitates (1) and (2) in water gave a peroxidase reaction with guaiacum[1] and hydrogen peroxide, but the reaction was stronger in the case of the latter showing that it contained most peroxidase. Since, however, only fraction (1) was able to oxidise nitrite, it follows that itate and peroxidase are not identical, and that they

[1] Made by dissolving 0·5 g. of guaiacum resin in 5 cc. of absolute alcohol, filtering and diluting with an equal volume of water. The solutions of this reagent used throughout this work were always freshly prepared and ascertained to be free from peroxide by testing against fresh milk.

can be separated by making use of their different solubilities in half-saturated ammonium sulphate. These facts are illustrated by the following experiment:

Exp. 2. 900 cc. of whey were half-saturated with ammonium sulphate and the precipitate, fraction 1, so obtained, was filtered off on á Buchner funnel and redissolved in 200 cc. of water. The filtrate was completely saturated with ammonium sulphate and the second precipitate, fraction 2, filtered off and also dissolved in 200 cc. of water. To 50 cc. of the solutions of each fraction, containing 1 cc. of 10 % acetic aldehyde, were added respectively 1, 2, 3 and 4 cc. of sodium nitrite[1] and the eight bottles were then rotated in the thermostat at 45°. After 20 minutes fraction 1 had oxidised 2 cc. of nitrite and after 60 minutes almost completed the oxidation of 3 cc. but was not able to effect the oxidation of 4 cc. even after 180 minutes. Fraction 2 on the other hand, after 90 minutes, had not appreciably diminished the amount of nitrite in any of the bottles. It is therefore clear that itate activity is confined to the precipitate obtained by half-saturating whey with ammonium sulphate.

It will be noted from the above experiment that the activity of the itate is considerably reduced by the process of precipitation with ammonium sulphate and re-solution in water when compared with its activity in whey. Thus, whereas 75 cc. of whey were able to oxidise 10·5 cc. of nitrite, the solution of fraction 1, obtained from 225 cc. of whey, in 50 cc. of water, could only oxidise between 2 and 3 cc., in other words approximately one tenth of that oxidised by the same volume of whey.

The inactivation of itate and peroxidase.

It was pointed out in the earlier paper [1923, p. 678] that the termination of itate activity coincides with the disappearance of the peroxidase reaction, and that when once a given quantity of nitrite has been oxidised further added nitrite is no longer attacked, even though the amount of milk employed contains sufficient itate to oxidise a much larger quantity of nitrite.

It is now found, however, that the oxidation of a further quantity of nitrite can be effected, provided it is added before the complete oxidation of that already present. These facts suggested that during the course of the reaction some product might be formed which had a destructive action upon both the itate and the peroxidase, rendering them inactive as soon as all the nitrite is oxidised. If this were so it followed that the peroxidase would not be inactivated so long as the nitrite were kept in excess. That this assumption is correct was shown by the following experiment:

Exp. 3. 75 cc. of milk and 1 cc. of 10 % acetic aldehyde were rotated with 16 cc. of sodium nitrite, a quantity known to be in excess of the amount which the itate present could normally oxidise[2]; it was found that, after

[1] The concentration of the sodium nitrite used throughout these experiments was 0·182 %.

[2] The largest volume of nitrite we have observed to be oxidised, using 1 cc. of acetic aldehyde, is 15 cc. of sodium nitrite, although on earlier occasions the maximum was 10 cc. in 210 minutes. We have however, using 2 cc. of acetic aldehyde, oxidised 17 cc. of nitrite in 240 minutes, but we were not able to increase this quantity by the addition of more aldehyde. No great significance must be attached to the absolute values since it is reasonable to expect a certain amount of variation in different specimens of milk.

5½ hours' rotation, the mixture still contained unchanged nitrite but gave a positive reaction with guaiacum and hydrogen peroxide, indicating the presence of unchanged peroxidase, whereas a quarter of an hour's rotation of milk with aldehyde alone was sufficient to inactivate all the peroxidase.

The fact that in this case peroxidase and nitrite could exist together without mutually interacting even after the addition of more aldehyde, suggested for the first time that the peroxidase alone was not responsible for the oxidation of nitrite.

The function of the aldehyde in the reaction.

In attempting to explain the function of the aldehyde in assisting in the oxidation of the nitrite, it was clear that it could not be that of a hydrogen acceptor in a "hydrolytic oxidation-reduction," leaving the oxygen from the water to oxidise the nitrite, since, in that case, no function could be assigned to the atmospheric oxygen which had already been shown to be essential to the reaction [Haas and Hill, 1923].

In view of the known tendency of benzaldehyde to form, with atmospheric oxygen, a peroxide, the possibility of a similar peroxide formation from acetic aldehyde was indicated.

To test this matter further, 75 cc. of water were rotated at 45° with 1 cc. of 10 % acetic aldehyde. No trace of peroxide formation could be detected after four hours' rotation, on addition of a few drops of guaiacum solution and a little fresh milk to supply the peroxidase. When however the 75 cc. of water were replaced by an equal volume of milk, it was found that, after rotating for a quarter of an hour, the mixture no longer gave any reaction for peroxidase but gave a strong positive reaction for peroxide when tested as above with guaiacum and milk.

Since milk alone when rotated in the thermostat produces no peroxide, it would appear that the production of a peroxide depends upon the interaction of acetic aldehyde with some constituent of the milk. Furthermore the essential part played by atmospheric oxygen in the reaction was shown by the fact that no peroxide was produced when milk and aldehyde were rotated in a bottle in which the air was replaced by nitrogen.

Subsequently it was found that a peroxide was also formed when acetic aldehyde was replaced by benzaldehyde but not by formaldehyde.

With regard to the particular constituent of milk concerned in the peroxide formation, two possibilities presented themselves, namely, itate and peroxidase. By rotating solutions of fractions (1) and (2), respectively, with acetic aldehyde, it was shown that the former, which contains itate and peroxidase, was alone able to produce peroxide, while the latter, which contains peroxidase only, gave no peroxide. This shows that it is the itate and not the peroxidase which is concerned in the formation of a peroxide.

THE MECHANISM OF THE OXIDATION OF NITRITE TO NITRATE.

In view of the above facts the conclusion appears justified that when milk is rotated in a thermostat, with acetic aldehyde and sodium nitrite, in a bottle containing air, the first reaction to take place is the formation of a peroxide from the aldehyde and atmospheric oxygen, through the intervention of the itate; the peroxide so formed then interacting with the peroxidase effects the oxidation of the nitrite.

The following equations are put forward tentatively in possible explanation of the above changes:

$$CH_3CHO + O_2 = CH_3CO \cdot O_2 \cdot H$$

$$CH_3CO \cdot O_2 \cdot H + NaNO_2 = CH_3COOH + NaNO_3$$

but it must be expressly stated that neither acetyl peroxide nor acetic acid has actually been isolated; in view however of the minute quantities of material involved the difficulty of doing so will be appreciated.

In support of the above theory of oxidation of the nitrite, by the interaction of peroxide with peroxidase, the following experiment may be quoted:

Exp. 4. Two bottles each containing 75 cc. of milk, 6 cc. 0·3 % hydrogen peroxide and 5 cc. of sodium nitrite were fitted up, one containing air and the other nitrogen. In each case the nitrite was destroyed in 15 minutes, showing that the oxidation depends upon the interaction between peroxide and peroxidase, and not upon the itate which, as was shown above, can only exert its action in the presence of oxygen.

That the oxidation of nitrite cannot be effected by hydrogen peroxide in the absence of peroxidase was shown by rotating a solution of these two substances in water instead of in milk, when no oxidation of nitrite was effected.

Since hydrogen peroxide in an atmosphere of nitrogen, is able to replace acetic aldehyde and oxygen, which by the action of itate produce a peroxide, it may be concluded that the chief function of the itate is to produce the requisite peroxide for interaction with the peroxidase, and that it is not itself concerned directly in the oxidation of the nitrite.

Further evidence for the essential part played by the peroxidase in the oxidation is furnished by the following experiment:

Exp. 5. 850 cc. of whey were divided into two portions, *A* and *B*. Portion *A* was half-saturated with ammonium sulphate and the precipitate was filtered off on a Buchner funnel and redissolved in 100 cc. of water giving solution *A* of fraction 1. Portion *B* was completely saturated with ammonium sulphate and the precipitate, consisting of fractions 1 and 2, was likewise dissolved in 100 cc. of water giving solution *B*. To 75 cc. of each of solutions *A* and *B* were added 1·5 cc. of 10 % acetic aldehyde and 1·5 cc. of sodium nitrite and the two mixtures rotated side by side in the thermostat. After 35 minutes the whole of the nitrite in solution *B* had been destroyed but that in solution *A* had not been destroyed after two hours. Furthermore solution *A*, after 35 minutes, contained no more peroxidase but gave a peroxide reaction, whereas solution *B* still gave a peroxidase reaction but was free from peroxide.

It must be borne in mind that both solutions *A* and *B* contained the same

quantity of itate (supplied by fraction 1) and only differed in their peroxidase content (the major portion of the peroxidase being contained in fraction 2). In view of these facts, the absence of peroxidase from solution *A* at the end of the reaction appears to indicate that the failure to oxidise the whole of the 1·5 cc. of sodium nitrite added was due to an insufficiency of peroxidase; on the other hand the excess of peroxidase found at the end of the reaction in solution *B* indicates that the peroxide formed by the itate was completely used up in oxidising the nitrite with the result that some unchanged peroxidase remained.

The action of peroxides upon itate and peroxidase.

To test the action of hydrogen peroxide on milk peroxidase 75 cc. of milk were rotated with 6 cc. of 0·3 % hydrogen peroxide; at the end of 40 minutes the peroxidase was found to have been entirely destroyed. From the above observation of the destructive effect of hydrogen peroxide upon milk peroxidase, the conclusion appears justified that it is the peroxide generated from acetic aldehyde which is responsible for the inactivation of the peroxidase when once all the nitrite present has been oxidised. It would appear therefore that the peroxide is utilised in the oxidation of the nitrite so long as the latter is in excess, but if insufficient nitrite is present the peroxide destroys the remaining peroxidase. Thus while 75 cc. of milk when rotated for 90 minutes with 1 cc. of 10 % acetic aldehyde and 16 cc. of sodium nitrite still contain unchanged peroxidase but no peroxide, the same quantity of milk rotated with 6 cc. of nitrite for 35 minutes no longer contains any peroxidase, but gives a positive reaction for peroxide.

The fact that the oxidation of nitrite has been shown to be due to the interaction between peroxide and peroxidase explains the failure to oxidise any further nitrite added to milk in which the peroxidase has already been inactivated.

Although the inactivation of peroxidase has been shown to be due to the peroxide produced by the action of itate on aldehyde, there was not sufficient experimental evidence to show whether itate itself is similarly inactivated by peroxide. The addition of more peroxidase to milk in which the peroxidase had been inactivated following on the oxidation of a quantity of nitrite should provide the requisite conditions for the further oxidation of added nitrite by the residual peroxide. If, in these circumstances, itate were still present in an active state, it should be possible to effect the oxidation of further nitrite up to the maximum of 15 cc.

It was shown in an experiment in which 6 cc. of sodium nitrite had been oxidised in 75 cc. of milk containing 1 cc. of aldehyde that, on the addition of fresh peroxidase in the form of fraction 2, it was only possible to oxidise an additional 3 cc. of nitrite. Since the total 9 cc. of nitrite thus oxidised is 6 cc. less than that capable of being oxidised by 75 cc. of milk it must be concluded that the activity of the itate is considerably reduced by the peroxide remaining at the end of the oxidation of the 6 cc. of nitrite.

Conclusions.

Without expressing any opinion on the chemical nature of itate it may be stated that its function in the oxidation of nitrite by milk in presence of acetic aldehyde appears to be an indirect one, inasmuch as it is concerned primarily in the production of the peroxide; that it is not directly responsible for the oxidation of the nitrite is shown by the fact that fraction 2, containing peroxidase but no itate, is able to oxidise nitrite when supplied with a peroxide in the form of hydrogen peroxide.

In effecting the oxidation of the acetic aldehyde to a peroxide the itate is apparently only able to produce a limited amount of peroxide and is itself used up in the process; in the absence of further experimental evidence its mode of action in this respect must remain a matter of speculation. In the meantime it cannot fail to be noted that in its effect it is similar to the hypothetical enzyme "oxygenase" which is assumed by Bach and his collaborators to be an essential constituent of the direct acting oxidase system of plants.

We have made a number of attempts to effect the oxidation of nitrite in the presence of acetic aldehyde and of hydrogen peroxide using the peroxidase of horseradish in place of that of milk but have not so far been able to obtain evidence of any oxidation.

Summary.

1. Itate and peroxidase, while sharing the common properties of being inactivated by boiling and of remaining in the whey on coagulation of milk, are not identical, since it is possible to effect a partial separation of the two substances.

2. The role played by itate in the oxidation of nitrite by milk in the presence of acetic aldehyde and oxygen, is that of producing a peroxide from the aldehyde. In this respect it resembles the hypothetical "oxygenase" of plant oxidases.

3. The oxidation of nitrite by milk results from the interaction of the peroxide so formed with the peroxidase, and does not result from the direct action of the itate, as originally thought. This is shown by the fact that a solution containing a mixture of itate and peroxidase will not oxidise any nitrite in the absence of peroxide, whereas a solution containing peroxidase, but no itate, does oxidise nitrite on the addition of hydrogen peroxide.

4. Attempts to effect a similar oxidation of nitrite by a peroxidase of vegetable origin, in the presence of hydrogen peroxide, have so far been unsuccessful.

During the course of this work we have kept in communication with Mr T. G. Hill who, from unavoidable causes, was prevented from active cooperation.

In conclusion one of us (B.L.) wishes to express her indebtedness to the Department of Scientific and Industrial Research for a grant.

REFERENCE.

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LXXXI. A REDETERMINATION OF THE HEAT OF COMBUSTION OF GLYCOGEN, WITH SPECIAL REFERENCE TO ITS PHYSIOLOGICAL IMPORTANCE.

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Introduction.

Although the heat of combustion of glycogen has already been determined by Stohmann and Schmidt [1894] and by Emery and Benedict [1911], it has now become desirable that a more accurate determination be made in order that this constant may be satisfactorily applied in the correlation of the work of Hill and Meyerhof on the physical and chemical phenomena accompanying muscular activity.

The previous determinations were both made with care and apparent accuracy so far as the actual combustion was concerned, but in neither case was the product used submitted to a satisfactory examination, in particular with respect to the state of hydration. Stohmann and Schmidt prepared their glycogen from the liver of a single rabbit, the extraction being made in the usual manner and the protein precipitated by the potassium mercury iodide method. The resulting product did not agree with the formula $(C_6H_{10}O_5)_n$ the discrepancy being attributed by the authors to the probable presence of fat. The sample was therefore extracted with ether and dried in an air oven at 110° to constant weight. The quantity of material was so greatly reduced by this process that there remained only sufficient for the determination of the heat of combustion, and in consequence no tests could be made to prove the purity of the sample. Harden and Young [1902] have shown that glycogen can only be rendered anhydrous by drying at 100° *in vacuo* over phosphorus pentoxide. Owing to the less efficient drying method used by Stohmann and Schmidt it is probable that the material employed by them for the final determination was not anhydrous glycogen as they assumed, and that in consequence their result of 4190·6 cals. per gram is almost certainly too low.

The belief that the above determination is too low is strengthened by the results of the determinations made by Emery and Benedict, who using samples of glycogen obtained from other workers calculated the heat of combustion

[1] The major portion of this work was carried out whilst working for the Medical Research Council.

per g. on the carbon content determined by ultimate analysis. Their figures 4214 and 4241 cals. are higher than the Stohmann and Schmidt figure, but owing to the fact that these workers did not prepare and purify the glycogen themselves, the results cannot be looked upon as reliable.

In order to obtain the value for the heat of combustion in dilute aqueous solution (the constant required for physiological purposes), it is necessary to correct both the above results for the heats of wetting, hydration and solution of anhydrous glycogen. The magnitude of these corrections is influenced by the state of hydration of the glycogen burnt, and in consequence cannot be applied in the results under consideration.

The present determinations have been carried out on samples of glycogen prepared from *Mytilus edulis* and *Ascaris lumbricoides*, the state of hydration of which had been definitely determined by drying and ultimate analysis. The heats of wetting and solution have also been found for the fully hydrated substance which was used in the combustion, and in this way a value has been obtained which represents the heat of combustion in dilute aqueous solution.

The result so obtained (3836 cals. per g. of hydrated glycogen $(C_6H_{10}O_5, H_2O)_n$ in dilute solution) is relatively higher than those given above and the values calculated from it and from the heat of combustion of lactic acid for the heat evolved during isometric contraction of muscle are in excellent agreement with the direct measurements of Hartree and Hill [1923]. A paper dealing with these relationships has already been published by the author [1923]. Since the publication of this paper it has come to his notice that the value for the heat of combustion of cane sugar assumed in the calibration of the bomb differed from that employed by Meyerhof [1922] in the corresponding determination on lactic acid. The value used by Meyerhof being the better authenticated [see Henning, 1921], and moreover for physiological purposes it being essential that the two determinations should be strictly comparable, a recalculation of the results was made using the same figure as that employed by Meyerhof, viz. 3952 cals. per g. of cane sugar.

The resulting correction in the value for the heat of combustion of glycogen from 3874 to 3836 does not materially affect the argument previously advanced in order to explain the source of the "delayed anaerobic heat" described by Hartree and Hill [1923]. The corrected figures are as follows:

Heat of combustion of 1 g. of glycogen monohydrate in dilute solution	3836 cals.
Heat of combustion of 1 g. of lactic acid in dilute solution [Meyerhof, 1922]...	3601 „
Heat available due to the formation of 1 g. of lactic acid from 1 g. of glycogen monohydrate...	235 „

If it is assumed that the whole of the lactic acid is immediately neutralised by alkaline salts, the total heat liberated can be calculated by using the

figure determined by Meyerhof [1922] for the neutralisation, viz. 19 cals. per g. of lactic acid, and is found to be 254 cals. per g. This is not in very close agreement with the figure given by Hartree and Hill of 296 cals. per g. of lactic acid formed. If it is assumed however that a portion of the lactic acid is neutralised in the first place by alkali protein, which Meyerhof has shown to have a heat of neutralisation of 138 cals. per g. of lactic acid, this difference is easily explained. When the whole of the lactic acid has been neutralised by alkali protein the heat available will be 235 + 138, *i.e.* 373 cals., which is in close agreement with the value, 370 cals., which is assumed (from Meyerhof) by Hartree and Hill for the total anaerobic heat.

It is only necessary therefore to alter the previous conception to the extent of assuming (*a*) a mixed neutralisation by salts and alkali protein immediately on the formation of the lactic acid, in the ratio of 74 % of salts and 26 % of alkali protein, instead of the complete initial neutralisation by salts, and (*b*) that the subsequent change from salt neutralisation to alkali protein neutralisation goes to completion instead of only to the extent of 60 % as was supposed in the previous paper.

The figure given on p. 629 for glycogen from *Ascaris lumbricoides* is the result of only one determination, no more available material remaining after the purity had been established.

Preparation and Purification of the Glycogen.

From Mytilus edulis. Two hundred mussels which had been sent alive from the Lancashire coast were used for each preparation. The shells were opened and the mussels thrown into a litre of boiling water. After it had been ascertained that the reaction of the liquid was slightly alkaline, the boiling was continued for half an hour. The mussels were then filtered off by means of a piece of muslin and the extraction repeated. The joint extracts were rendered faintly acid, and the boiling continued for two to three minutes to precipitate protein. The coagulated protein was filtered off and alcohol added until the solution contained 60 %. The glycogen settled out rapidly and the greater part of the supernatant liquid could be decanted off. Strong caustic potash solution was now added to the glycogen sludge remaining, until the whole solution contained 50 to 60 % of potash. The solution at this stage amounted in a normal experiment to 300 to 400 cc., a volume in which the glycogen readily dissolved. The solution was heated over a wire gauze for six hours, cooled and almost neutralised by strong acetic acid; the potassium acetate which separated out was filtered off, and finally the glycogen was reprecipitated by alcohol and filtered from the solution.

The rest of the purification consisted of the repeated solution of the glycogen in water and its reprecipitation by alcohol. As the electrolytes were gradually removed the precipitation became more difficult, and it was found sometimes to be advisable to add small traces of potassium acetate in order to facilitate the formation of a curd; this salt being appreciably soluble in the

alcohol-water mixtures used for precipitation remains in solution. It is of interest to note in connection with the solution of glycogen in water, that the rate of solution is not materially increased by heating but that continuous agitation is very effective in rendering the solution rapidly complete. A yield of 23 g. of dry glycogen was obtained from 200 mussels.

From Ascaris lumbricoides. The worms freshly taken from the pig's gut were cut into small pieces and thrown into ten times their weight of boiling water. The chopped worms were filtered off and the extraction repeated; the subsequent purification process was carried out exactly as in the case of the mussels. The yield was only 2 to 3 g. from 200 g. of worms.

Method used for drying the purified glycogen.

When the glycogen was judged to be sufficiently pure, as much as possible of the alcoholic mother liquor was decanted off, and the glycogen washed twice with 70 % alcohol. The wet glycogen so obtained was transferred to a flask and an excess of dry benzene added. After the mixture had been allowed to stand over night the alcohol-water-benzene fraction and the alcohol-benzene or water-benzene fraction as the case might be, were distilled off through a fractionating column, and the excess benzene remaining poured from the glycogen. (For further description of this method of drying see Atkins and Wilson [1915].) At the end of the distillation the glycogen had formed a hard pale brown vitreous cake, which, as the adhering benzene was removed in a gentle stream of air, broke up with a concoidal fracture into a coarse glistening powder. This powder was ground in a mortar to a flour-like consistency when it became almost pure white. The final product was quite free from benzene and in appearance dry, showing no tendency to form lumps or to adhere to glass.

Examination of the glycogen.

The samples obtained by the above process were examined, in each case where the quantity of material allowed, for ash, fat, nitrogenous impurities, and an ultimate analysis was made. The examination for water content proved so interesting that it is dealt with under a separate section.

The samples were all found to be free from fat. The nitrogenous impurities were estimated by Folin's micro-Kjeldahl method. The results obtained are given in Table I.

Table I.

Sample	Ash %	Nitrogen %	Ultimate analysis C %	H %
1st sample *Mytilus*	Nil	Trace	39·90	6·64
2nd sample *Mytilus*	0·02	Trace	39·83	6·53
Sample *Ascaris*	0·10	Trace		

Calculated for $(C_6H_{10}O_5)_n$, C = 44·44 % and H = 6·17 %.
Calculated for $(C_6H_{10}O_5, H_2O)_n$, C = 40·00 % and H = 6·66 %.

There was not sufficient *Ascaris* glycogen available for ultimate analysis.

STATES OF HYDRATION OF GLYCOGEN.

From the analytical results given above it was concluded that the sample of glycogen was in a hydrated form corresponding to the formula

$$(C_6H_{10}O_5, H_2O)_n.$$

This hydrate is called for convenience in the subsequent discussion the monohydrate.

A specimen of the monohydrate was dried over calcium chloride *in vacuo*. Water was lost rapidly at first, as will be seen from the accompanying curve, but later the velocity of dehydration fell off roughly exponentially until a steady state was reached with a total loss of 5·02 %.

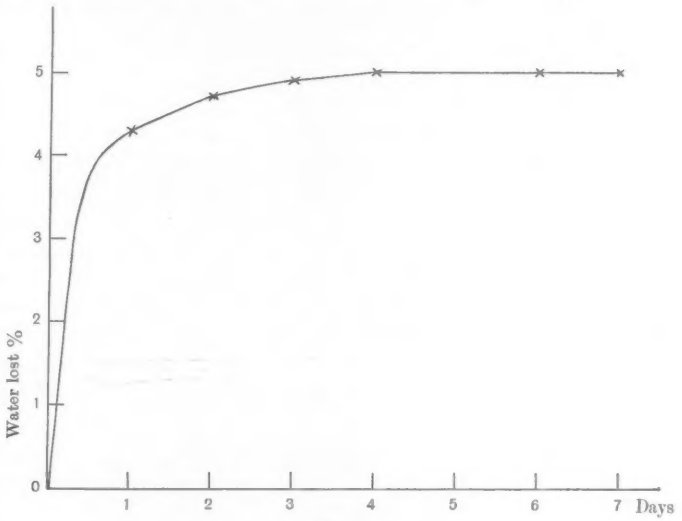

Fig. 1.

The curve in Fig. 1 shows the result of a typical experiment.

Approximately the same loss was found in all the experiments tried. The equation

$$2\,(C_6H_{10}O_5, H_2O)_n = 2\,(C_6H_{10}O_5, 1/2\,H_2O)_n + H_2O$$

requires a loss of 5·00 % of the total weight. It was therefore concluded that the drying over calcium chloride *in vacuo* results in the formation of a half hydrate. Bizio [1867] has already described this substance as the product obtained when wet glycogen is dried to constant weight over calcium chloride *in vacuo*.

When the half-hydrate was placed in an air oven at 110°, it commenced to lose weight immediately and very rapidly. After about one hour a sudden change in the rate of dehydration took place, and from this point the velocity

was extremely slow. The total loss gradually approached 10 % of the initial weight of the glycogen monohydrate.

These results are plotted in Fig. 2.

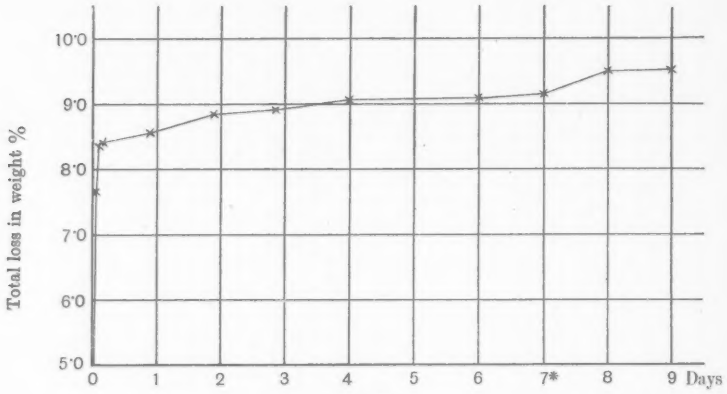

Fig. 2.

* The temperature of the oven was, at this point, raised to 120° C.

From these experiments it was concluded that sufficiently prolonged heating would produce anhydrous glycogen, but that the time necessary to do so would be approximately 15 days. Moreover, although in the present instance no decomposition was observed at 120°, it is probably unwise to use temperatures higher than 110°. It is clear from this experiment that the flat portion of the curve may be easily mistaken for the steady anhydrous state, whilst the glycogen still contains from 1·5 to 2·0 % of water.

Glycogen which is nearly anhydrous is definitely hygroscopic. A sample containing 0·5 % of water, on standing exposed under ordinary laboratory conditions for 35 hours was found to contain 4·2 % of water.

Determination of the heats of wetting and solution of the monohydrate.

Method. The apparatus used for the determination consisted essentially of an ordinary Dewar vessel from a "thermos" flask, immersed up to the neck in a thermostat which was kept at 25°.

The flask was closed by a cork carrying a thermometer graduated to 0·01°, a heating coil which could also be used for calibration purposes, and an arrangement for containing and releasing the glycogen. The latter consisted of a small specimen tube in which the glycogen was placed. The tube was supported by a rubber band resting on a copper wire ring, through which the tube could just move with ease. A piece of stout cotton thread was attached to the bottom of the tube so that when a steady temperature state had been obtained, the tube could be lifted and upset by pulling the cotton thread. This method

insured the immediate wetting of the whole of the glycogen. It was found necessary to attach another piece of cotton thread to the upper rubber ring in order to free the tube if it stuck whilst it was being raised through the copper holder. The copper ring was held in the cork by means of a short length of glass tubing, so that the heat leak through the cork might be reduced to a minimum.

In order to mix the contents, the flask was gently shaken from time to time. A control experiment was made in order to ascertain whether this procedure produced any material change in the temperature gradient. The results showed that within reasonable limits the shaking had no effect.

100 cc. of water were used in the calorimeter in two of the experiments whilst in the third 200 cc. were used, the equivalent of the whole calorimeter being redetermined. The difference in the amount of water used caused no difference in the results.

Calibration. The water was weighed into the flask and the cork placed in position. A current was then passed through the heating coil until the temperature inside the flask was within 0·2° of the temperature of the thermostat. The apparatus was then left to stand for an hour at the end of which time the temperature had become nearly steady. The change in temperature, if any, was observed over a period of 15 minutes. At the end of the initial temperature readings the heating circuit was closed for an accurately measured period, and temperature readings taken at intervals of one minute until a steady fall was observed. The necessary corrections were made for the cooling during the experiment and the actual rise in temperature calculated. The heating current was measured with an ammeter, and the heat calculated in the usual way. Table II gives the results of the calibration.

Table II.

Number of experiment	Rise in temperature (° C.)	Weight of water (g.)	Total heat equivalent (cals.)	Heat equivalent of calorimeter (cals.)
1	1·139	100·1	114·0	13·9
2	1·219	100·2	117·9	17·8
3	1·258	100·0	116·1	16·1
4	0·592	200·1	214·2	14·1

Average value for the equivalent of the calorimeter = 15·5.

Results of determination. The procedure given above was followed in each case, and the results are given in Table III.

Table III.

Number of experiment	Rise in temperature (° C.)	Weight of water (g.)	Total heat equivalent * (cals.)	Weight of glycogen (g.)	Heats of wetting and solution (cals. per g.)
1	0·082	99·2	114·7	1·1294	8·3
2	0·116	99·6	115·1	1·3880	9·6
3	0·026	198·7	214·2	0·6444	8·6

Average value = 8·8 cals. per g.

* Neglecting the dissolved glycogen.

Determination of the heats of hydration, wetting, and solution of the half hydrate.

Two measurements were made of these values using the same technique as for the monohydrate. The results obtained were as follows:

Table IV.

Number of experiment	Rise in temperature (° C.)	Weight of water (g.)	Total heat equivalent * (cals.)	Weight of glycogen (g.)	Heats of hydration, wetting and solution (cals. per g.)
1	0·143	97·5	113·0	1·3240	12·2
2	0·063	97·6	113·1	0·6180	11·6

* Neglecting the dissolved glycogen.

The weights of glycogen given are the calculated weights of the monohydrate formed from the half-hydrate taken, so that the results represent the heat given out in the formation of 1 g. of monohydrate and its subsequent solution.

Determination of the heat of combustion of the monohydrate.

Method. A Mahler-Cook bomb calorimeter was used for the determination of the heat of combustion. The established technique was employed with very slight variations to suit the particular case.

The most important variation introduced was in the arrangement of the material and the method of firing. The author has found from past experience that it is much more satisfactory to place the substance to be burnt directly in the crucible without briquetting or wrapping in a collodion envelope. The combustion under these conditions takes place evenly and rapidly, and without spurting. There is, however, a little more difficulty in the ignition, but this can be overcome by stretching the platinum ignition wire tightly between the leads, and allowing a small piece of cotton thread, tied at one end to the wire, to dip into the substance in the crucible. Such an arrangement rarely gives a misfire, and the heat given out by the thread can be corrected for, if a definite weight of cotton is used for each experiment. A good cotton yarn is sufficiently uniform to permit of a measured length being used for each experiment.

The following possible sources of error were considered and it was concluded that they could be neglected.

(1) The heat evolved by the passage of the current. A number of test experiments were made with the platinum ignition wire exposed to view and it was found that the making of the current and the fusion of the wire were, as far as could be observed, simultaneous. The needle of a delicate ammeter in the circuit was not disturbed. Under these circumstances the heat evolved must be negligible.

(2) The heat changes due to the burning of any nitrogen present in the oxygen used. The nitrogen present in the quantity of oxygen used for the combustion was not sufficient to require an appreciable amount of heat if it

were completely burned. As the reaction is endothermic any small error would be opposite in effect to a similar error due to the passage of the current as described above.

(3) Inaccuracies in the thermometer. The thermometer was a standard instrument supplied with the bomb, and was found to show no noteworthy irregularities.

(4) The change in the specific heat of water with change of temperature is not sufficient to affect the results obtained at ordinary temperatures.

Calibration of the bomb. The bomb was calibrated by burning saccharose. The saccharose contained no moisture or ash and gave on ultimate analysis C = 41·9 % and H = 6·40 %. (Calculated C = 42·10 % and H = 6·43 %.) The heat of combustion of saccharose was taken as 3952 cals. per g. The calibration determinations gave the following figures:

Table V.

Number of experiment:	1	2	3	4
Weight of saccharose (g.)	1·9550	1·9394	1·8616	1·2020
Heat available from saccharose (cals.) ...	7726·2	7664·5	7357·0	4750·3
Heat available from cotton (cals.) ...	8·2	8·2	8·2	8·2
Total heat available (cals.)	7734·4	7672·7	7365·2	4758·5
Rise in temperature (° C.)	2·370	2·355	2·257	1·462
Heat required per ° C. rise (cals.) ...	3263·5	3258·1	3263·3	3254·8
Weight of water used (g.)	2500·9	2499·9	2500·0	2497·0
Heat equivalent of calorimeter (cals.) ...	762·6	758·2	763·3	757·8

Average value = 760·5 cals. per ° C. rise.

Determination of heat of combustion of monohydrate. Six determinations were made on two samples, *M* 1 and *M* 2, of *Mytilus* glycogen, and one on the sample (*A*) of *Ascaris* glycogen. The following table gives a summary of results:

Table VI.

Number of experiment:	1	2	3	4	5	6	7
Source of glycogen	*M* 1	*M* 1	*M* 1	*M* 2	*M* 1	*M* 2	*A*
Weight of glycogen (g.)	1·2510	1·1111	1·0377	1·1161	0·8322	1·0302	0·5036*
Rise in temperature (° C.)	1·478	1·310	1·229	1·319	0·982	1·217	0·595
Weight of water in calorimeter (g.)	2500	2501	2500	2503	2501	2500	2498
Heat equivalent of calorimeter (cals.)	760·5	760·5	760·5	760·5	760·5	760·5	760·5
Total heat equivalent (cals.)	3260·5	3261·5	3260·5	3263·5	3261·5	3260·5	3258·5
Heat from cotton (cals.)	8·2	8·2	8·2	8·2	8·2	8·2	8·2
Heat evolved from glycogen (cals.)	4810·8	4264·4	3999·0	4296·4	3194·6	3959·8	1930·6
Heat of combustion per g. (cals.)	3845·6	3837·7	3853·7	3849·5	3838·7	3843·7	3833·6

* After allowing for ash.

Result and conclusions. Considering only the determinations made on the *Mytilus* glycogen the average value for the heat of combustion of the monohydrate is 3844·8 cals. per g. The *Ascaris* value is omitted as the purity of the specimen was not so definitely established, the result being regarded merely as an indication that glycogen from a second source gives a value of the same order as that from *Mytilus*.

If the value for the heat of wetting and solution of the monohydrate (8·8 cals. per g.) is subtracted, the heat of combustion of glycogen in dilute solution is found to be 3836 cals. per g. This value is lower than that previously given by the author, for reasons stated in the introduction, but is considerably higher than the corresponding values of Stohmann and Schmidt, and of Emery and Benedict. Such a deviation from the previous results is to be expected in view of the difficulty experienced in obtaining anhydrous glycogen, and suggests in the case of Stohmann and Schmidt that the glycogen used contained 1·5 to 2·0 % of water.

Summary.

(1) The previous determinations of the heat of combustion of glycogen are criticised on the grounds of the doubtful state of hydration of the samples of glycogen used, and the fact that they do not give the heat of combustion of dissolved glycogen (which is required for physiological purposes), but only of glycogen in the anhydrous state.

(2) A monohydrate of glycogen of the formula $(C_6H_{10}O_5, H_2O)_n$ can be prepared by fractionally distilling off with benzene the water and alcohol used in its preparation. This hydrate on drying over calcium chloride *in vacuo* yields a half-hydrate $(C_6H_{10}O_5, 1/2\ H_2O)_n$. The anhydrous material is very difficult to obtain, and is produced only by drying at 110° for about 15 days.

(3) The total heat of wetting and solution of the monohydrate is found to be 8·8 cals. per g., and the total heat of hydration, wetting and solution of the half-hydrate to be 11·9 cals. per g.

(4) The heat of combustion of the dry monohydrate is found to be 3844·8 cals. per g., for the average of six determinations on two samples of *Mytilus* glycogen, and 3833·6 cals. per g. in one determination on *Ascaris* glycogen.

(5) The heat of combustion therefore of glycogen monohydrate in dilute solution, taking only the *Mytilus* figure, is 3836 cals. per g.

(6) The value now given is less than that used by the author in his paper dealing with the application of this constant to the theory of muscular contraction [1923], but the change does not materially affect the argument given there. It is required only to assume that the initial neutralisation of lactic acid is carried out partly by alkali protein instead of entirely by alkaline salts, and that in the final state the neutralisation is entirely due to alkali protein, instead of only to the extent of 60 per cent.

The author wishes to thank Prof. A. V. Hill, Mr A. D. Ritchie and Dr A. L. Robinson for the help and encouragement they have given during the progress of the research. The expenses of the research have been largely borne by a grant from the Royal Society.

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LXXXII. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. II.

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(*Received April 15th, 1924.*)

This communication describes a number of further experiments in which antiscorbutically active fractions have been obtained from lemon juice. In the previous contribution of this series [Zilva, 1924] it was shown that it was possible to remove the acids and sugar from juice without perceptibly influencing its potency. These substances, as is well known, form the great bulk of the solid matter of the juice. The active residue left after the removal of the acid and sugar usually comprises only about $\frac{1}{30}$th part of the total solids in spite of the fact that it contains the entire activity of the equivalent quantity of lemon juice. This active residue was submitted to further fractionation, utilising various precipitating methods, and the writer is now able to record results which show, that it is possible to remove still more extraneous matter without vitiating the activity of the antiscorbutic principle; in fact preparations have been obtained containing only ·03–·07 % of solids whilst retaining the same activity as the original juice. This was demonstrated by adhering to the practice of testing out every preparation quantitatively.

As was mentioned in the last communication, the sugar can be removed from the decitrated lemon juice by fermentation, leaving an active fraction consisting of an apparently complex mixture of substances and showing an appreciable nitrogen content with a total solids content of 0·3–0·5 %. When this fermented active juice is concentrated to a small volume and treated with absolute alcohol a precipitate is formed. This precipitate is not active, and it can be shown that the entire activity remains in the supernatant fluid. Basic lead acetate also forms a precipitate when added to fermented decitrated lemon juice, but in this case the active principle goes entirely into the precipitate. By combining these two procedures, *i.e.* by precipitating with absolute alcohol first, distilling off the alcohol and adding basic lead acetate to the active aqueous solution, a very potent preparation is obtained on removing the lead from the precipitate.

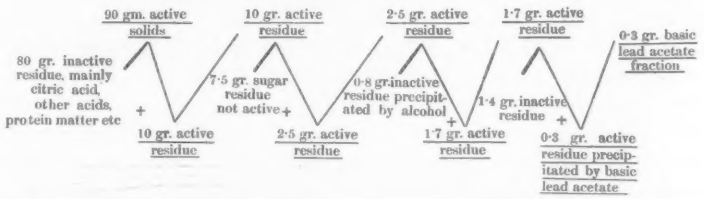
Attempts were made to elaborate a method which would render possible the production of such an active fraction with less labour. In the first place it was found that basic lead acetate brings down the vitamin entirely from decitrated lemon juice before fermentation. In this case, however, some sugar

and other non-active substances are also removed in the precipitate and, therefore, this treatment does not yield as pure a fraction as the one described above. Secondly, it was found that the procedure of decitrating the lemon juice could be modified in a way which conduces to economy in labour and materials and which at the same time yields a juice more free from non-active matter than that obtained by the original method.

When such decitrated juice is precipitated with basic lead acetate and the precipitate freed from the lead, the residue after evaporation taken up in a small volume of water and treated with absolute alcohol in order to remove some inactive matter by precipitation, an active fraction is obtained of considerable purity but still containing sugar.

The purest fraction has been obtained by fermenting juices decitrated by the modified method, and submitting the fermented solutions to alcohol and basic lead acetate precipitations as previously described. The following table gives the scheme of fractionation. Every fraction contains the entire antiscorbutic activity of a litre of lemon juice.

Table I.


90 gm. active solids
80 gr. inactive residue, mainly citric acid, other acids, protein matter etc
+
10 gr. active residue
10 gr. active residue
7·5 gr. sugar residue not active
+
2·5 gr. active residue
2·5 gr. active residue
0·8 gr. inactive residue precipitated by alcohol
+
1·7 gr. active residue
1·7 gr. active residue
1·4 gr. inactive residue
+
0·3 gr. active residue precipitated by basic lead acetate
0·3 gr. basic lead acetate fraction

The above figures are an approximate average. They vary to some extent with the juice.

Experimental.

All the fractions were tested out for their activity on guinea-pigs. The activity of the ultimate fraction obtained in this investigation was also confirmed on monkeys. The guinea-pigs were kept on the usual scorbutic diet of oats, bran and a limited quantity (40 cc.) of autoclaved milk. The preparations to be tested were administered daily. The dosing commenced after the animals had been about 14 days on the scorbutic diet and was continued in the case of positive tests until two calendar months had elapsed from the time the animals were placed on the diet. Only in two sets was this period reduced to 54 days. All the fractions were freshly prepared either daily or every other day. In the latter case the preparation was acidified and kept in a vacuum over night. Daily doses equivalent to 1·5 cc., 3 cc. and 5 cc. of the original lemon juice were tried in every case. The lowest of these doses may be considered the approximate minimum daily dose of lemon juice capable of protecting a guinea-pig from scurvy for the above period. The animals were

regarded as protected when at the end of the two months only slight signs or no signs of scurvy were found after chloroforming them.

Exp. 1. Fermented decitrated juice was concentrated to about one-sixth of its volume, and about ten times its bulk of absolute alcohol was gradually added. A precipitate was formed which was centrifuged and dissolved in a quantity of water equivalent to the original juice. The alcohol was distilled from the supernatant fluid in a vacuum at 50°. This fraction was also made up to the equivalent volume of the original juice. The solution of the precipitate when tested out for its antiscorbutic activity was found not to be able to prevent or even delay scurvy in guinea-pigs in daily doses of 1·5 cc., 3 cc. and 5 cc. It did not reduce ammoniacal silver nitrate in the cold. The solution from which this precipitate was removed when tested on two animals in each of the above doses was found in every case to prevent the onset of scurvy for two months. This solution reduced ammoniacal silver nitrate in the cold and decolorised potassium permanganate. As an average four Kjeldahl determinations gave 1·5 cc. $N/50$ NH_3 for an equivalent of 10 cc. of original juice.

Exp. 2. Basic lead acetate was gradually added to fermented decitrated juice until no more precipitate was formed. The solution was then quickly centrifuged and the residue dissolved in acetic acid. Magnesium sulphate was then added until all the lead was precipitated, and the solution was treated with two volumes of absolute alcohol. After filtering, the alcohol and acetic acid were removed by distillation in a vacuum at 50° and the preparation was brought up to the equivalent volume of the original juice. The lead could not be removed by hydrogen sulphide as the resulting preparation was found to be toxic to the animals. It was also not considered advisable to use sulphuric acid for the purpose, as even a small excess of acid left in the solution would have had a harmful effect on the animals, whilst any excess of magnesium sulphate could only, at its worst, act as a purgative. Two pigs were employed on each of the doses in this experiment. The animals on the 1·5 cc. succumbed to scurvy after about 40 days. The 3 cc. and 5 cc. doses protected the animals for two months. The slight loss in the antiscorbutic activity in this experiment was undoubtedly due to imperfect manipulation. This active fraction gives a less intense Pauli reaction, reduces ammoniacal silver nitrate in the cold and decolorises potassium permanganate. As an average four Kjeldahl determinations gave 0·5 cc. $N/50$ NH_3 for an equivalent of 10 cc. of original juice.

The filtrate from the above precipitate was freed from lead as described above. The resulting solution was tested on six guinea-pigs in the usual doses. All the animals succumbed to scurvy in 21–30 days. The entire active principle was, therefore, removed by the precipitate.

Exp. 3. In this experiment the procedures employed in Exps. 1 and 2 were combined, *i.e.* after removing the inactive residue from fermented decitrated juice by alcohol precipitation as described in Exp. 1, the solution was precipitated with basic lead acetate until no further precipitate was obtained. The precipitate was then treated as in Exp. 2. Three animals were used for

each dose. All the nine guinea-pigs were protected from scurvy for two months by the administration of doses of 1·5 cc., 3 cc. and 5 cc. of this fraction. This shows that no loss in activity was incurred by this manipulation. The average solid matter content of this preparation was found to be ·05–·07 %. It reduced ammoniacal silver nitrate in the cold and decolorised potassium permanganate. As an average of six Kjeldahl determinations an equivalent of 10 cc. gave 0·3 cc. $N/50$ NH_3. In three phosphorus estimations equivalents of 100 cc. of the original juice gave ·000172, ·000175 and ·000141 g. P. It gave an almost negative Pauli reaction.

Exp. 4. Decitrated lemon juice was treated with basic lead acetate without being previously fermented. An active fraction fairly free from extraneous matter was thus obtained but, as was to be expected, the lead acetate precipitated under these conditions, together with the vitamin, more inactive matter, including sugar in varying quantities, than when the juice was treated as in Exp. 3. The sugar content of this fraction is, however, only about one-tenth of that of the original decitrated juice. Three guinea-pigs receiving 1·5 cc. doses, two, 3 cc. doses and four, 5 cc. doses were protected from scurvy for two months by the administration of this preparation.

Exp. 5. In decitrating the lemon juice of the above experiments the method described on several previous occasions was used. It consisted in neutralising the lemon juice with an excess of chalk, treating the neutralised solution with two or three volumes of absolute alcohol, filtering and removing the alcohol by distillation in a vacuum. This procedure can be advantageously modified. The lemon juice is treated with excess of chalk as before and allowed to remain for 30–60 minutes. This enables salts to deposit. It is then filtered and the filtrate concentrated in a vacuum at 50° to about a fifth of the original volume. A volume of alcohol equal to about half the original volume of juice is gradually added. The precipitate is then filtered off, washed with a little alcohol, and the alcoholic washings are combined with the filtrate, the alcohol distilled off in a vacuum at 50° and the residue made up to the original volume. By this modification not only are alcohol and time saved but an active preparation is obtained from which more of the non-active matter is removed than by the old method. Tested out on six guinea-pigs, it was found that 1·5 cc. was the minimum protective dose for two months.

Exp. 6. Lemon juice decitrated as in Exp. 5 was precipitated with basic lead acetate. The active precipitate was dissolved in acetic acid, treated with magnesium sulphate and two volumes of alcohol and concentrated to a small bulk. It was then treated with excess of alcohol. The gummy precipitate formed by this treatment was centrifuged and the supernatant solution after removing the alcohol by distillation was made up to the original volume. Four guinea-pigs on a dose of 1·5 cc., two on a dose of 3 cc. and three on a dose of 5 cc. were protected from scurvy for 54 days. The preparation contains less non-active matter and sugar than the one obtained in Exp. 4. It reduces ammoniacal silver nitrate in the cold and decolorises potassium permanganate.

As an average of six Kjeldahl determinations an equivalent of 10 cc. gave 0·5 cc. $N/50$ NH_3. Three phosphorus estimations carried out on an equivalent of 100 cc. gave 0·0000166 g., 0·000049 g., 0·0000543 g. P. It gave a very faint Pauli reaction.

Exp. 7. This fraction was prepared in the same way as that of Exp. 3 with the difference that the decitrated lemon juice from which it was precipitated was prepared as in Exp. 5. A purer fraction was obtained by this method, the total solids of which came to 0·03–0·05 %. As an average of six Kjeldahl determinations an equivalent of 10 cc. gave 0·2 cc. $N/50$ NH_3. Two phosphorus estimations carried out on an equivalent of 100 cc. gave 0·000137 g. and 0·0000909 g. P. It reduced ammoniacal silver nitrate in the cold and decolorised potassium permanganate. It gave a very faint almost negative Pauli reaction. Daily doses of 1·5 cc., 3 cc. and 5 cc. were found to protect guinea-pigs from scurvy for 54 days. This fraction was also tested out curatively on monkeys. The monkeys were first kept on a scorbutic diet of boiled rice, marmite, salts, caseinogen and cod liver oil for about seven weeks, when they developed scurvy. This same diet was continued throughout the experiment.

Protocol.

Monkey A. Developed pseudoparalysis, very well marked ulceration of the gums in the vicinity of upper incisors on 23. iii. 24. Received amount of the preparation dissolved in water equivalent to 100 cc. of the original juice at 1 p.m. on the same day. On 24. iii. 24 improvement in the pseudoparalysis was observed, but proptosis and orbital haemorrhages had developed since the first symptoms were observed. Received another equivalent of 100 cc. of lemon juice at 10 a.m. on that day. Gradual improvement followed and by 28. iii. 24 the animal had recovered.

Monkey B. Developed very marked proptosis, orbital haemorrhages and pseudoparalysis on 25. iii. 24. Received an equivalent of 100 cc. of lemon juice. Gradual improvement. On 28. iii. 24 received an equivalent of 50 cc. of lemon juice. By 31. iii. 24 all symptoms of scurvy disappeared.

Discussion.

Two points emerge from this investigation which deserve comment. The first is the very low nitrogen and phosphorus content of the purest fractions. The above experiments, however, do not offer enough evidence to show whether these elements are associated with the active principle or not. In the case of the nitrogen averages are given, but it is to be pointed out that cases were met in which nitrogen could not be found at all in the fraction as prepared in Exp. 7. This is rather suggestive of the possibility that the active principle may not contain any nitrogen. The subject is now being followed up.

The second point of interest is that all the active fractions reduce ammoniacal silver nitrate in the cold and decolorise potassium permanganate. This has

been studied in some detail [Connell and Zilva, 1924], and evidence is now available that an absolute parallelism between the reducing substance or substances and the vitamin does not exist.

SUMMARY.

A series of experiments is described in which some very active antiscorbutic fractions have been prepared. The most potent preparation contains only ·03–·07 % of solid matter in solution whilst retaining approximately the entire activity of the original lemon juice. All the active fractions reduce ammoniacal silver nitrate and decolorise potassium permanganate. The purest preparations showed an extremely low nitrogen and phosphorus content.

The author wishes to express his thanks to the Medical Research Council for a whole time grant.

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LXXXIII. THE REDUCING PROPERTIES OF ANTISCORBUTIC PREPARATIONS.

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(*Received April 29th, 1924.*)

In the preceding communication [Zilva, 1924] mention was made of the property of antiscorbutic fractions to reduce ammoniacal silver nitrate in the cold and to decolorise potassium permanganate. The following experiments show that in spite of the close association of the two properties this reducing substance, or substances, is inactivated at a different rate from the antiscorbutic factor. One may therefore conclude that part at least if not all of this reducing material is chemically independent of the physiological principle.

The destruction of the reducing properties of decitrated lemon juice was studied by boiling the juice in the presence and in the absence of air and by exposing it to the action of the atmosphere at ordinary temperature in alkaline medium.

In the first experiment the juice was adjusted to p_H 6·6–6·8 and heated in a boiling water-bath under a reflux condenser. In the aerobic experiments air was aspirated through the boiling solution. In the anaerobic experiments a current of carbon dioxide gas was passed during the heating.

In the second experiment the juice was made $N/20$ alkaline and treated in the way already described [Zilva, 1923].

For the reduction of the potassium permanganate 20 cc. of a 0·5 % solution of the reagent and 10 cc. of 4 N sulphuric acid were added to 0·5 cc. of decitrated lemon juice. The reduction proceeded slowly but it was found that a period of 24 hours was sufficient to complete it. The flasks were kept in tha laboratory at ordinary temperature and additional permanganate was added if necessary. The residual potassium permanganate was titrated with sodium thiosulphate.

In the silver reduction experiments 5 cc. of $N/14$ ammoniacal silver nitrate solution were added to the decitrated lemon juice and allowed to remain for 20 minutes (reduction is complete after 15 minutes). A crystal of barium nitrate and 4 cc. of $N/10$ nitric acid were added to agglutinate the reduced

silver (p_H 7·2–7·4). After filtering off the precipitate a further 5 cc. of $N/10$ nitric acid was added and the unreduced silver nitrate was titrated with $N/10$ sodium chloride, using potassium chromate as an outside indicator. It was found that the square of the amount of silver reduced (expressed in cc. of the solution) was proportional to the volume of the decitrated lemon juice used, and the constant obtained by the ratio of the former to the latter was used as a measure of the amount of reduction. This is illustrated by the following figures:

	Volume of decitrated juice taken cc.	Amount of reduction in cc. $N/10$ $AgNO_3$	$\frac{(\text{Amount of reduction})^2}{\text{Vol. of solution taken}}$
I.	1	·3	·09
	2·5	·5	·10
	5	·7	·10
	10	1·2	·14
	20	1·6	·12
II.	2·5	·8	·25
	5	1·2	·29
	10	1·7	·29
	20	2·0	·20

Tables I and II give the details of the experiments.

Table I. *Inactivation by heat.*

Solution	Percentage of silver-reducing substance destroyed	Percentage of permanganate-reducing substance destroyed
Original	0	0
Heated in CO_2 for 3 hours	0	0
Heated in air for 1 hour	25±15	5
Heated in air for 3 hours	40±20	5

Table II. *Inactivation by alkalinity.*

Solution	Percentage of silver-reducing substance destroyed	Percentage of permanganate-reducing substance destroyed
Original	0	0
Standing 24 hours in vacuum	0	0
Standing 1 hour in air	30±12	6
Standing 3 hours in air	45±25	6

It will be seen that the material responsible for the decolorisation of the potassium permanganate is very little affected by either treatment. A bigger impression was made on the silver reducing substance, although a marked variation is recorded in this respect in the various samples. Even the highest figure, however, is considerably lower than that obtained in the inactivation of the antiscorbutic factor under similar conditions [Zilva, 1922, 1923].

In this connection reference may be made to the observation recorded by Bezssonoff [1922] that certain antiscorbutic substances produce a blue coloration with a modified Folin's reagent for phenol, which is evidently a reduction process. He suggested that this reaction might be utilised as a test for the

antiscorbutic factor. Kay and Zilva [1923] have since shown that certain antiscorbutic preparations failed to give this reaction whilst substances such as yeast which, as is well known, do not possess antiscorbutic properties, produce the coloration. Bezssonoff [1924] in a subsequent communication modifies his view and asserts that the coloration is produced by a degradation product of the antiscorbutic factor and that if an active substance fails to give the reaction directly it will do so after heating. He also criticised Kay and Zilva's observation on the behaviour of yeast towards the reagent, asserting that his improved reagent [1923] does not give a coloration with yeast.

We have repeated the experiments with the yeast, using the improved reagent, and, as before, we obtained a coloration. Heating the adsorbed antiscorbutic preparation used by Kay and Zilva on the other hand again failed to give the colour. Further active fractions as obtained in Exps. 3, 6 and 7 [Zilva, 1924] also gave negative results before and after heating. The reaction is therefore useless for the purpose of establishing the presence of the antiscorbutic factor.

Thanks are due to the Medical Research Council for a whole time grant.

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LXXXIV. THE DIFFERENTIAL DIALYSIS OF THE ANTISCORBUTIC FACTOR. II.

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(*Received April 15th, 1924.*)

Adopting the technique of Brown [1915], Zilva and Miura [1921] were able to show that the antiscorbutic factor of lemon juice diffuses through collodion membranes which permit the passage of substances of such molecular dimensions as methylene blue, neutral red and safranine. The vitamin of course was also found to diffuse through collodion membranes of higher permeability. In extending the investigation the authors hoped to utilise this method of differential dialysis for the purpose of separating the active principle from extraneous matter. Although the original intention could not be accomplished, other interesting information was obtained which is recorded in this communication.

The three main observations made in this investigation are:

(A) That the diffusion of the antiscorbutic factor proceeds differently from that of the sugar and nitrogenous substances present in decitrated lemon juice, which confirms the chemical observations made on the juice in this connection [Zilva, 1924, 1, 2].

(B) That the antiscorbutic vitamin in another source, namely swede juice, seems to diffuse through membranes of the same permeability as when it is present in lemon juice. This would suggest that the factor in both sources is more or less of the same molecular magnitude.

(C) That the vitamin will diffuse through collodion membranes of somewhat lower permeability than those which permit the passage of the dyes mentioned above.

Experimental.

The technique employed in this investigation was with the exception of a few modifications the same as that used by Zilva and Miura, which as already mentioned was based on that of Brown. The principle consists in soaking the dry collodion thimbles in alcohol of various strengths. The stronger the alcohol employed the greater the permeability obtained. The collodion used was a 14 % alcohol-ether solution (Necol 356/A9) to which 100 cc. of ether per litre were added. In order to obtain as great an accuracy as possible the viscosity

of the collodion was kept constant by frequent measurements and additions of ether if necessary. The thimbles were soaked in the respective alcohols for 24 hours at a constant temperature of 20° in an incubator. The alcohols were made up by weight in the following concentrations:

Table I.

S. G.		% by weight	Approx. % by volume
·812	hydrometer	92·2	95
·8235	weighing bottle	88·1	92
·8280		85·8	90
·8340		83·8	88·5
·840	hydrometer	81·4	87

Exp. 1. As has already been shown the sugar in decitrated lemon juice consists mainly of invert sugar [Zilva, 1924, 1]. It was thought desirable to ascertain whether the diffusion of invert sugar in aqueous solution proceeds in the same way as that of the sugar in decitrated lemon juice. Decitrated juices, prepared by the old method of treating the neutralised lemon juice with two volumes of alcohol before concentrating, showed an average sugar content as estimated by Bertrand's reduction method of 0·45 %. The pure sugar solutions were therefore made up by using equimolecular mixtures of glucose and fructose giving a final mean concentration of 0·47 % estimated by the same method. The dialysis of the sugar in decitrated lemon juice and of the sugar in aqueous solution was compared on twelve occasions. The duration of dialysis in each case was three days. The following table gives the mean figures of the twelve experiments.

Table II. *Percentage of sugar diffused.*

Alcohol index of membrane ...	95 %	92 %	88·5 %	87 %
Decitrated juice	97	49	9	0
Sugar solution	97	61	20	0

It will be seen from Table II that there was a deviation in the diffusibility of the sugar in the decitrated juice from that in the aqueous solution through the 92 % and 88·5 % membranes. The differences are beyond the limits of the experimental error of the method. It can, however, be shown that this deviation is due to the blocking of the pores of the membranes by matter in solution in the decitrated juice and not to any difference in the actual molecular dimensions. When the decitrated juice was diluted with three times its volume of water it was found that the diffusion of the sugar proceeded as in the case of the aqueous solutions of glucose and fructose. This is seen from Table III.

Table III. *Percentage of sugar diffused.*

Alcohol index of membrane	88·5 %	90 %	92 %
Decitrated juice, original strength	7	21	50
Decitrated juice, diluted four times	18	43	61
Sugar solution	20	—	61

The experiment, therefore, shows that the sugar diffuses in the same way in both cases.

Exp. 2. In this experiment the relative quantities of the antiscorbutic factor, the sugar, and nitrogenous substances diffusing in three days through membranes of various permeability were studied; 95 %, 92 %, 90 %, 88·5 % and 87 % membranes were employed. A new series of the various thimbles containing about 20 cc. of the juice was placed in the dialysing tank every other day, so that no juice was fed to the guinea-pigs more than two days after dialysis. Some juice in a glass tube was kept in the dialysing tank with each batch as a control. Some succinic acid was introduced in each thimble to keep the juices on the acid side. Succinic acid was chosen on account of its low solubility in water and its non-toxic properties.

The antiscorbutic factor could not of course be estimated with the same degree of precision as the sugar and the nitrogen. The accuracy obtained by the biological method was, however, found to be good enough to demonstrate the different degrees of diffusion of the three fractions. Three doses equivalent to 1·5 cc. 3 cc., and 5 cc. of the original lemon juice were used in estimating the vitamin in the decitrated lemon juice dialysed through each membrane and in the control juice. Two animals were used for each dose in the case of the 95 % membranes. All the six animals succumbed to scurvy within 30 days, showing that the whole of the vitamin had diffused. A similar set of animals was used for the 92 % series. All these animals died of scurvy, those on the lower doses within a month and those on the two higher doses within 35–38 days. It is, therefore, concluded that about 95 % of the vitamin has passed through this membrane. In the case of juice dialysed in the 90 % thimble the assessment was rather more difficult to make. Of the three animals on the daily dose of 1·5 cc. one died of florid scurvy within 35 days, the other two within 52 and 53 days respectively, showing signs of moderate scurvy at the post-mortem examination. On the 3 cc. dose one animal died within 56 days, showing marked scurvy, and another had to be chloroformed 46 days after the commencement of the experiment owing to intercurrent disease. This animal was also scorbutic. The animals on the 5 cc. dose survived for two months after which time they were chloroformed. Some signs of scurvy were observed at the post-mortem. It is estimated from the above that about 40 % of the vitamin diffused in these thimbles. The animals fed on the juice from the 88·5 %, 87 % membranes and from the control tubes all behaved in the same way. The animals on 1·5 cc. doses succumbed to scurvy, but after a delay of about 20 days. The animals on the 3 cc. and 5 cc. doses survived for two months after which time they were chloroformed. The similarity in the behaviour of the animals in these three sets shows that no appreciable quantity has diffused through the last two membranes. Table IV and Fig. 1 give the results of these experiments.

Table IV. *Percentage of substances diffused.*

		%	%	%	%	%
Sugar	Alcohol index of membrane	87	88·5	90	92	95
Mean of 12 estimations by Bertrand's method	Mean value	0	9	21	50	97
	Mean variation	0	±2·5	±4·5	±4	0
Nitrogen						
Mean of 11 estimations by a micro-Kjeldahl method	Mean value	10	25	50	70	100
	Mean variation	±5	±10	±10	±7·5	—
Antiscorbutic factor		0	0	40	95	100

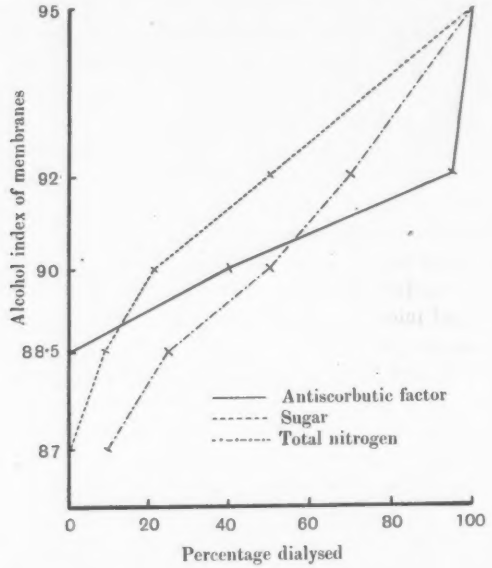

Fig. 1.

In analysing the results it is seen that the nitrogenous substances of the decitrated juice diffuse at different rates to the antiscorbutic factor, and this lends further support to the view that the vitamin is independent of the bulk of the nitrogenous matter. The sugar figures being an index of a definite substance and not of a probable mixture of substances as is the case of the nitrogen figures, definitely confirm the previous observation [Zilva, 1924, 1] that the invert sugar has no connection with the antiscorbutic activity. It is further seen that the 87 % membrane does not allow the passage of the sugar and the vitamin whilst the 88·5 % membrane permits the slow passage of the former but no passage of the latter. This would suggest that the dimensions of the active molecule and that of the hexoses cannot be far removed.

Exp. 3. This experiment was instituted with the intention of studying the rate of diffusion of the antiscorbutic factor in swede juice. The results were partly vitiated by certain unforeseen technical difficulties but they contribute enough information to be recorded. It was found that the animals

receiving the doses did not thrive well and mostly died before the end of the experimental period, probably through the action of some toxic principle which was produced in the juice during dialysis.

The swede juice was dialysed in the same way as the decitrated lemon juice. Swede juice as obtained from the root by pressing contains some matter in colloidal suspension even after filtration. It was, therefore, not advisable to dialyse it in this condition as there was a likelihood that the pores of the dialysing thimbles might be blocked by this matter and the results thus vitiated for comparative purposes. The juice was, therefore, previously treated with an equal volume of absolute alcohol which precipitated most of the colloidal matter. After filtration the alcohol was removed in a vacuum at 50° and the juice brought up to its original volume with water. Preliminary experiments had shown that this precipitation did not affect the potency of the juice. The minimum protective dose in the case of both the precipitated and the original juice was found to be 3 cc.

Table V gives a summary of the results obtained with the dialysed swede juice.

Table V.

Alcohol index	Dose	Days alive	Findings at P.M. examination
80 %	3	47	Very mild scurvy: death not due to scurvy alone
	5	46	Scurvy
	7	39	Death not due to scurvy
90 %	3	40	Very mild scurvy: death not due to scurvy alone
	5	54	Mild scurvy
	7	24	Death not due to scurvy
95 %	3	26	Died of scurvy
	5	24	„ „
	7	26	„ „

Reviewing these results one is inclined to conclude, in spite of the imperfections of the technique, that the 80 % and 90 % membranes did not allow the vitamin to diffuse to any considerable extent, since definite protection against scurvy was observed even in the animals receiving the lowest dose which affords protection when the undialysed juice is fed immediately after preparation. The intermediate doses showed even better protective power. No doubt can exist as to the inefficiency of the swede juice which was dialysed through a 95 % membrane.

It would, therefore, appear that the antiscorbutic factor of this source diffuses through membranes of the same permeability which permit its passage when present in lemon juice.

Summary.

The diffusion of the antiscorbutic factor proceeds differently from that of the sugar and the nitrogenous substances present in decitrated lemon juice.

The antiscorbutic factor in swede juice and in lemon juice diffuses through membranes of the same permeability.

Indications are obtained which suggest that the size of the active molecule cannot be far removed from that of the hexoses.

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LXXXV. THE DISSOLUTION OF GELATIN.

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(*Received April 23rd, 1924.*)

In a previous communication under this title by Fairbrother and Swan [1922] evidence was put forward to show that when gelatin is put into water or dilute acid or alkali, some of the gelatin goes into solution in the liquid surrounding the gel. This dissolved gelatin was estimated by precipitation with tannic acid. No experimental evidence was there put forward to show the particular molecular complexity of the substance in solution, although it was suggested that the phenomenon of dissolution was not principally due to hydrolytic decomposition of the gelatin.

Several workers have criticised the conclusions arrived at in the above paper. Liesegang [1923] states that the solution (*i.e.* cold water which has stood in contact with gelatin) can be strongly concentrated by careful evaporation without forming a jelly, and hence that the solution consists only of hydrolysis products of gelatin.

Knaggs, Manning and Schryver [1923] come to the conclusion that the dissolution of gelatin in water is chiefly, if not entirely, due to thermal degradation and goes on continuously over many months, and hence that the 10 days allowed by Fairbrother and Swan [1922] did not represent an equilibrium time. Liesegang, and Knaggs, Manning and Schryver consider only the dissolution in water.

Alexander [1923] after quoting the results of Fairbrother and Swan states: "They should be repeated with ash-free gelatin properly freed from products of hydrolysis, for it is probable that only the hydrolysis products dissolve in cold water."

In the present paper experimental evidence is given to show that whatever be its ultimate fate as regards hydrolysis, gelatin dissolves spontaneously to some extent, in water, dilute acid or dilute alkali in a form which can be recovered and made into a jelly again.

Experimental.

A sample of Coignet's Gold Label Gelatin was used which was the same as "B" in the work carried out by Fairbrother and Swan.

One gram quantities were put in sterile flasks with 100 cc. water and a little thymol, and the flasks tightly stoppered. The flasks were kept at about 20° in an electrically heated incubator, and occasionally gently shaken.

Flasks which had stood thus for 7, 10, 32 and 42 days respectively were removed, the contents filtered and 3 to 4 times the volume of redistilled acetone added to each. In each case a milky white precipitate was produced which settled slowly. After standing for 2 to 3 days this was filtered off, dried, and 1 cc. cold water added. The precipitate which was in the form of a greyish powder swelled quickly to a loose, wrinkled gelatinous mass on the filter paper. This was liquefied by warming and poured into a small tube. On cooling the sol set to a turbid gel which was quite firm at room temperature in the case of 7, 10 and 32 days experiments and slightly less firm in that of 42 days.

A similar experiment was performed with 1 g. Nelson's Photographic Gelatin No. 1 which was allowed to stand for 7 days in 100 cc. of water at 20°. This also gave a precipitate with acetone, which when made into a 5 % sol with water, formed a firm gel on cooling.

Hence, the gelatin which goes into solution in water at 20° can, to some extent at least, be recovered as gelatin.

These experiments show that the dissolution in water is not primarily a thermal degradation or breakdown of the gelatin molecule.

With a view to a re-examination of the time factor as a consequence of the findings of Knaggs, Manning and Schryver [1923] determinations were made of the dissolution of this same Gold Label Gelatin, and of another sample of the same brand which had been purified by the method of Miss Field [1921] followed by precipitation with alcohol. The dissolution experiments were carried out at 20°. The experimental details of the estimations were as given by Fairbrother and Swan.

The values given below are the mean of two determinations.

Time in days	Weight of precipitate in g. given by tannic acid with 25 cc. of solution	
	Unpurified gelatin	Purified gelatin
4	0·027	0·027
7	0·029	0·034
14	0·040	0·042
21	0·047	0·049
42	0·053	0·050

These figures show an increase with time, of the gelatin dissolved, although, if the weight of precipitate be plotted against the time, the curve appears to be approaching a limiting value.

It is to be expected that in any case some slow continuous increase with time of the dissolution will take place, just as a jelly will continue to swell in water for many months although the major part of the swelling has taken place within the first few days. The "maximum" of dissolution is probably of a similar nature to the "maximum" swelling of a gel.

It should be pointed out that the above experiments were carried out at 20° as were those of Knaggs, Manning and Schryver.

The experiments of Fairbrother and Swan were performed at a lower temperature, at which one would expect any thermal degradation to be less marked.

It is probable that a small rise of temperature causes a more rapid physical, and reversible disintegration of the micellae rather than a chemical molecular decomposition. This breaking up of the micellae will increase very rapidly as the solation temperature is approached.

The above figures, however, do not show any marked difference in behaviour as between the commercial and the purified gelatin. Knaggs, Manning and Schryver state that gelatin which has been treated with dilute hydrochloric acid, and washed, does not yield as much "diffusible nitrogen" as untreated gelatin.

Further evidence that the dissolution during the first few days is not merely solution of degradation products already present in the gelatin, is given by experiments at a lower temperature. The amount of precipitate obtained by the addition of tannic acid as before to 25 cc. of water, 100 cc. of which had stood in contact with 1 g. of gelatin in a refrigerator (0°–2°) was as follows:

Days:	4	7	32
Weight of precipitate g.	0·005	0·005	0·005

In connexion with the dissolution in dilute acid and alkali the following experiments were carried out.

One g. of Gold Label Gelatin was placed, with a little thymol in 100 cc. $N/10$ hydrochloric acid and kept at 20°. After 10 days the gelatin, which had dissolved completely, was neutralised with sodium hydroxide, leaving the reaction a little on the acid side of the isoelectric point. This solution was allowed to stand in the laboratory for three days, when it was found to have set to a very weak jelly. The concentration of dry gelatin was about 0·8 %. The jelly was melted, the gelatin precipitated by acetone, dried and made up into a 5 % sol. This set to a stiff jelly on cooling, indicating that, in accord with experiments of Miss D. J. Lloyd [1920], gelatin does not dissolve in dilute hydrochloric acid as a consequence of a molecular breakdown.

It has, however, also been found possible to recover gelatin from solution in sodium hydroxide, although the mechanism of dissolution is clearly different from the former case. Two g. of Gold Label Gelatin allowed to dissolve in 100 cc. $N/10$ sodium hydroxide for three days at 20° when neutralised with N hydrochloric acid did not form a gel on standing. The gelatin was then precipitated by alcohol. The precipitate was of quite a different character from that yielded by a solution in hydrochloric acid precipitated by alcohol; it was more gelatinous in nature and could only with great difficulty be filtered. It was centrifuged, washed with alcohol, dried and made into a 2·5 % sol. This formed a gel on cooling. Acetone was found to be much better than alcohol as a precipitant; the precipitate was not gelatinous and was more easily separated.

In another experiment 1 g. of gelatin dissolved in 100 cc. $N/10$ NaOH at 20° in 48 hours. The solution was filtered and neutralised with hydrochloric acid using methyl red as indicator, one drop of a solution of sodium hydroxide was then added. The final reaction was well on the alkaline side of the isoelectric point, *i.e.* the indicator was quite yellow. The gelatin was

precipated by acetone and the precipitate made into a sol of about 5 %, which set to a firm gel on cooling. Hence the recovery of gelatin from solution in alkali is not entirely a question of the degree of neutralisation as suggested by Wilson [1923], but probably depends rather on the time the gelatin is left in contact with the alkali, since solutions of gelatin in alkali which have stood for longer than a few days, give on neutralisation and precipitation, products which yield progressively weaker gels.

It appears, therefore, that when a gel is put into dilute acid or alkali, gelatin can exist as such, in the outer liquid in equilibrium with the gel. If a Donnan membrane equilibrium is set up in such a case, as the work of Loeb, Proctor and others indicates, then it follows that the concentration of ionised gelatin within the gel must be greater than without. The present work makes it appear unlikely that this is caused by outer layers of gelatin acting as a semi-permeable membrane preventing the diffusion of the gelatin ions within the gel. A Donnan equilibrium would be set up if the gelatin cations were held from diffusing in some other way than by such a semi-permeable membrane. This makes it appear probable therefore that the gelatin cations are held as integral parts of the framework of the gel, which consists therefore, not of isoelectric gelatin alone, as postulated by Miss Lloyd, but also of aggregates of gelatin ions, which latter also exist in solution in the interstitial spaces within the gel and in the liquid without the gel.

An approximate calculation of the effect upon the Donnan equilibrium, which would be caused by the dissolved gelatin, shows that the effect would be very small.

Summary.

1. Gelatin dissolves on standing in contact with water, dilute acid, or dilute alkali at ordinary temperatures primarily as gelatin. Some subsequent hydrolysis probably occurs.

2. The framework of a gelatin gel must consist not only of isoelectric gelatin but also of gelatin cations.

The author wishes to take this opportunity to express his thanks to Messrs Brunner Mond whose research grant to this Department has facilitated the work on the study of gels.

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LXXXVI. THE INFLUENCE OF VARIATIONS IN THE SODIUM-POTASSIUM RATIO ON THE NITROGEN AND MINERAL METABOLISM OF THE GROWING PIG.

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The experiments recorded in this paper were carried out as part of an investigation being conducted at this Institute on the mineral requirements of the growing pig. Certain results, not yet published, obtained by some of our co-workers engaged in this investigation, seemed to indicate that the addition of sodium to a ration of cereals led to increased rate of growth. The following metabolism experiments were conducted with the object of determining the influence of the addition of sodium salts to a cereal ration on the assimilation and retention of nitrogen and of those minerals which are most essential as constructive material for the growing pig.

Previous Work.

Previous work on the effect of sodium and potassium salts in nutrition appears to have been almost entirely restricted to two questions, viz. (*a*) the effect of the addition of common salt in the feeding of farm animals, which forms one of the oldest subjects of agricultural research, and (*b*) the effect of increase of potassium salts on the excretion of sodium and chlorine, a question investigated by Bunge [1873], on the basis of whose one-day experiments the theory has been built up and generally accepted that intake of potassium salts leads to impoverishment of the organism in sodium chloride.

(*a*) *Effect of sodium chloride in animal feeding.* After various contradictory results recorded in the earlier part of the 19th century, Voit [1860] found that the addition of salt increased to a small extent the assimilation of nitrogenous substances, and this view was adopted for many years. Later researches, however, seem to show that its influence on nitrogen assimilation depends on the quantity in which it is taken. According to Belli [1904], who gives also a summary of the earlier literature on the subject, in the small amounts which are naturally present in foodstuffs it has a favourable influence, while in the quantities in which it is usually added to the food the result is unfavourable.

(*b*) *The effect of increase of potassium salts.* In his experiments on himself Bunge found that by taking doses of potassium in the form of the phosphate, the citrate or the sulphate, there was a very large increase in the excretion of sodium and chlorine in the urine. In a converse experiment, by taking a dose of sodium citrate, he found similarly an increase in the urinary excretion of potassium, the excretion of chlorine on the other hand showing a slight diminution. Two points are specially to be noted in regard to Bunge's work: (1) he analysed only the urine, no attention being paid to faecal excretion, and (2) his own figures show that the excessive loss of sodium and chlorine on the day of the experiment was made up for on the three days following the potassium intake, when the excretion of these elements was far below the normal. It is further noteworthy that Bunge himself, in summarising his work and mentioning future experiments that must be carried out, refers specially to the necessity for analysing the intestinal excretions, and for determining whether repeated intake of potassium salts would lead to continued excessive excretion of sodium and chlorine.

In spite of these admissions, and of the limited nature of his own experiments, Bunge maintains his point that the organism is impoverished in sodium chloride by the intake of potassium salts, and he proceeds to elaborate a theory that the craving for salt experienced by herbivorous animals and peoples [Bunge, 1874] is due to the large amount of potassium salts contained in vegetable foodstuffs, the excess of potassium leading to excessive excretion of sodium chloride, which has to be made good by increased intake of salt with the food, while on the other hand carnivorous animals shew no such craving.

In this connection it is perhaps worthy of mention that Köppe [1900] in discussing the salt craving shown by herbivorous animals, points out the difference between the ash-content and the salt-content of plants. He states that, in spite of their high ash-content, plants contain only a very small amount of dissolved and dissociated inorganic salts. The salts present are mostly in organic combination, while animal fluids on the other hand have a high content of free inorganic salts. This may also have a bearing on the fact that herbivorous animals, such as cattle and sheep, may be fed for a long time on potassium-rich food without any addition of salt, and show no visible disturbance in their development and condition. As Abderhalden [1923] points out, it is quite possible and indeed probable that the potassium salts of foodstuffs exercise no such strong influence as is brought about by the introduction into the body of potassium salts in large quantities in the inorganic form.

H. G. Miller [1923] investigated the influence of potassium on urinary sodium and chlorine excretion, his paper being published after the present series of experiments had been begun. His object was to find the effect of *continued* large additions of potassium to the diet, his experiments being carried out on a pig, but, like Bunge, he analysed only the urine, so that his results can lead to no conclusions as to the impoverishment of the organism

in sodium chloride. But, as a matter of fact, he finds that even in the urine alone, the increased sodium excretion in only transitory, in spite of continued administration of potassium.

Effect of Sodium and Potassium Salts on Calcium Assimilation.

Bunge's theory was taken up by Seemann [1879] who believed that he had found in it the cause of rickets. He argues thus: excessive supply of potassium causes excessive excretion of chlorine and hence want of chlorides in the body; this leads to insufficient formation of hydrochloric acid in the stomach, and owing to this deficiency of free acid the calcium salts of the food are not sufficiently dissolved in the stomach, thus leading to rickets. Zander [1881] followed up Seemann's work by investigating cases of rickets in breast-fed children. He found that in the case of healthy children the mother's milk contained potassium and sodium in the proportion of 2 : 1, while in the case of rachitic children the ratio was 3–4½ : 1. Hence Zander considers Seemann's theory fully confirmed.

The only other reference found to the effect of sodium and potassium salts on calcium assimilation is that of Aron [1905], who states: "From researches undertaken to investigate licking disease in cattle, it appears very probable that the content of the food in potassium and sodium influences the deposit of calcium and the bone formation, viz., with strongly diminished sodium content of the food, and at the same time very high potassium content, the deposit of calcium, and also the bone growth, remains below normal, in spite of abundant supply of calcium and phosphorus." No details are given by Aron, and the later paper which he promises on the subject seems never to have been published.

Present Investigation.

While the primary object of the present investigation was to ascertain the effect on calcium assimilation of altering the sodium-potassium ratio in an animal's diet, it seemed advisable also to study the effect on the general metabolism, and at the same time to find whether Bunge's conclusions were justified.

Methods.

The experiments were carried out on young pigs by the methods described in a previous paper by the present authors on the influence of oils on the assimilation of calcium and phosphorus in the growing pig [Husband, Godden and Richards, 1923] but in addition to determining the total nitrogen, calcium and phosphorus in the urine and faeces, there were also determined magnesium, chlorine, sodium and potassium; magnesium by the volumetric method of Tisdall and Kramer [1921]; chlorine by the Volhard method; potassium by the cobaltinitrite method of Green [1912]; sodium and potassium as mixed sulphates, and hence sodium by difference.

Experimental Data.

Basal diet. The basal diet used in the two experiments to be described consisted of maize, oatmeal, barley meal and blood meal in the proportions stated below. Analyses of the foodstuffs gave the following figures (averages of the samples used in the two experiments):

Average composition of the basal diet.

Proportions		Total N %	CaO %	P_2O_5 %	MgO %	Cl %	Na_2O %	K_2O %
Maize	10 parts	1·46	0·007	0·645	0·193	Nil	0·040	0·500
Oatmeal	10 „	2·19	0·082	1·068	0·232	Nil	0·067	0·618
Barley meal	10 „	1·22	0·056	0·718	0·164	0·007	0·061	0·659
Blood meal	1 part	13·37	0·141	0·301	0·037	0·934	1·196	0·414

The exact intake of nitrogen, CaO and P_2O_5, etc., in each experiment is stated in the tables giving the experimental data.

The amount of CaO in the ration was increased in each experiment by the addition of 25 cc. of a 20 % solution of calcium chloride, and in Exp. 2 it was still further increased by adding 6 grams of chalk. In addition the animal received 10 cc. of olive oil daily, the previous work having shown that this assisted regular defaecation, and helped to keep the animal in good condition. Any effect of the olive oil on assimilation would be continuous throughout the experiment, and may therefore be excluded in considering the results.

Exp. 1. *To determine the effect of the addition of sodium chloride to the ration.* In this experiment, which extended over 19 days, the animal received 775 g. of the basal ration plus 25 cc. of 20 % calcium chloride solution per day. From the 6th to the 12th day 50 cc. of a 10 % solution of sodium chloride were added to the daily ration, this being withdrawn again as from the 13th day. The results of the analyses are given in Table I and shown in part in Fig. 1. In order to economise space, only the averages for the periods are given in the tables, but it may be mentioned that the variations in the day to day figures are small.

It will be seen that the results of the administration of sodium chloride point to a definite increase in the rate of retention of the chief constructive materials required for proper growth. The balances for N, P_2O_5 and CaO, which had been falling during the preliminary period, all showed an increase during the experimental period. The results for MgO, given in the tables, are not meanwhile discussed, but it may be stated that, in general, the curves obtained resemble those for phosphorus.

With regard to the excretion of K_2O, the interesting result is noted that, while the feeding of sodium chloride caused a large increase in the urinary excretion of K_2O, as observed by Bunge, this was accompanied by a large decrease in the faecal excretion, so that the balance for the experimental period remained practically the same as in the pre-period, and in the post-period showed a distinct increase (see Fig. 1). This point will be referred to again in the discussion of the results after Exp. 2.

Table I. *Exp.* 1. *Average daily excretions and balances in grams.*

Sodium chloride experiment. Duration of experiment: 19 days.

Days	Average daily wt. of faeces	Nitrogen Intake	Urine	Faeces	Total	Balance	CaO Intake	Urine	Faeces	Total	Balance
1–5 inclusive	329	15·15	6·74	3·62	10·36	4·79	1·74	0·07	1·56	1·63	0·11
→ 6–12 „	289	„	6·71	3·27	9·98	5·17	„	0·08	1·23	1·31	0·43
← 13–19 „	276	„	6·96	3·20	10·16	4·99	„	0·06	1·12	1·18	0·56
		P_2O_5					MgO				
1–5 „		5·94	1·28	3·63	4·91	1·03	1·49	0·06	1·34	1·40	0·09
→ 6–12 „		„	1·62	3·22	4·84	1·10	„	0·06	1·29	1·35	0·14
← 13–19 „		„	1·51	3·22	4·73	1·21	„	0·05	1·26	1·31	0·18
		Cl					Na_2O				
1–5 „		1·97	2·50	0·10	2·60	−0·63	0·53	0·31	0·05	0·36	0·17
→ 6–12 „		4·84	4·82	0·12	4·94	−0·10	3·03	2·32	0·20	2·52	0·51
← 13–19 „		1·97	2·20	0·11	2·31	−0·34	0·53	0·45	0·11	0·56	−0·03
		K_2O									
1–5 „		4·38	0·59	2·47	3·06	1·32					
→ 6–12 „		„	1·31	1·83	3·14	1·24					
← 13–19 „		„	0·83	2·06	2·89	1·49					

→ indicates the introduction of sodium chloride.
← indicates the withdrawal of sodium chloride.

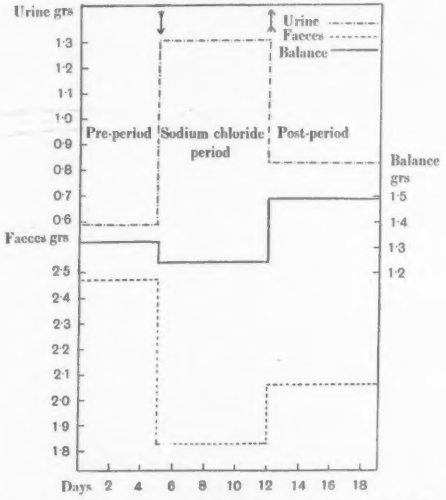

Fig. 1. K_2O excretions and balances. Experiment I, Table I.

Exp. 2. *To show the effect of the addition of sodium citrate to the ration.*

As there was a possibility that the improved balances in the experiment just recorded might have been due to the chlorine and not to the sodium of the salt administered, in this experiment sodium citrate was added to the

basal ration instead of sodium chloride. As before, the animal received daily 775 g. of the basal ration, with 25 cc. of 20 % calcium chloride solution and 6 g. of chalk. In the experimental period it received in addition, per day, 50 cc. of a solution of sodium citrate containing approximately the same amount of Na_2O as the sodium chloride solution of Exp. 1. In this experiment the periods were lengthened—preliminary period 16 days, the results being given for the last eight days of the period; experimental and post periods 14 days each. The results of this experiment are given in Table II and Figs. 2 and 3, and it will be seen that they confirm those of the first experiment.

Table II. *Exp.* 2. *Average daily excretions and balances in grams.*

Sodium citrate experiment. Duration of experiment: 36 days.

Days (inclusive)	Av. daily weight of faeces	Nitrogen					CaO				
		Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
1–4	260	15·88	6·27	3·87	10·14	5·74	5·26	0·39	2·56	2·95	2·31
5–8	282	,,	6·26	3·93	10·19	5·69	,,	0·45	2·60	3·05	2·21
→ 9–15	281	,,	6·05	3·87	9·92	5·96	,,	0·46	2·43	2·89	2·37
16–22	252	,,	6·06	3·38	9·44	6·44	,,	0·68	2·00	2·68	2·58
← 23–29	216	,,	6·22	3·03	9·25	6·63	,,	0·65	1·80	2·45	2·81
30–36	202	,,	5·95	3·12	9·07	6·81	,,	0·48	1·83	2·31	2·95
		P_2O_5					MgO				
1–4	—	6·36	0·27	3·42	3·69	2·67	1·48	0·11	1·23	1·34	0·14
5–8	—	,,	0·29	3·58	3·87	2·49	,,	0·11	1·27	1·38	0·10
→ 9–15	—	,,	0·30	3·61	3·91	2·45	,,	0·09	1·32	1·41	0·07
16–22	—	,,	0·29	3·19	3·48	2·88	,,	0·14	1·24	1·38	0·10
← 23–29	—	,,	0·23	2·93	3·16	3·20	,,	0·12	1·19	1·31	0·17
30–36	—	,,	0·22	2·94	3·16	3·20	,,	0·13	1·21	1·34	0·14
		Cl					Na_2O				
1–4	—	2·34	2·54	0·18	2·72	−0·38	0·91	0·52	0·49	1·01	−0·10
5–8	—	,,	2·51	0·20	2·71	−0·37	,,	0·42	0·49	0·91	+0·0
→ 9–15	—	,,	2·56	0·19	2·75	−0·41	3·44	1·79	1·11	2·90	+0·54
16–22	—	,,	2·49	0·18	2·67	−0·33	,,	2·19	1·17	3·36	+0·08
← 23–29	—	,,	2·56	0·15	2·71	−0·37	0·91	0·70	0·61	1·31	−0·40
30–36	—	,,	2·52	0·17	2·69	−0·35	,,	0·48	0·50	0·98	−0·07
		K_2O									
1–4	—	4·71	1·42	1·53	2·95	1·76					
5–8	—	,,	1·34	1·74	3·08	1·63					
→ 9–15	—	,,	2·26	1·24	3·50	1·21					
16–22	—	,,	2·05	1·20	3·25	1·46					
← 23–29	—	,,	1·35	1·40	2·75	1·96					
30–36	—	,,	1·36	1·52	2·88	1·83					

→ indicates the introduction of sodium citrate.

← indicates the withdrawal of sodium citrate.

Nitrogen, calcium and phosphorus assimilation. The balances for N, CaO and P_2O_5 in both experiments all show an increase during the experimental period, the increases continuing into the post-period, except that for N in Exp. 1, which fell back slightly. In Exp. 2, where the longer periods enable the results to be followed more closely (see Fig. 2), it will be seen that the improvements are all more marked during the second week of the sodium feeding, and that the increases fall off gradually in the post-period.

Urinary excretion of CaO. A noteworthy point in Exp. 2 was the very marked effect of the sodium citrate on the urinary excretion of CaO, which

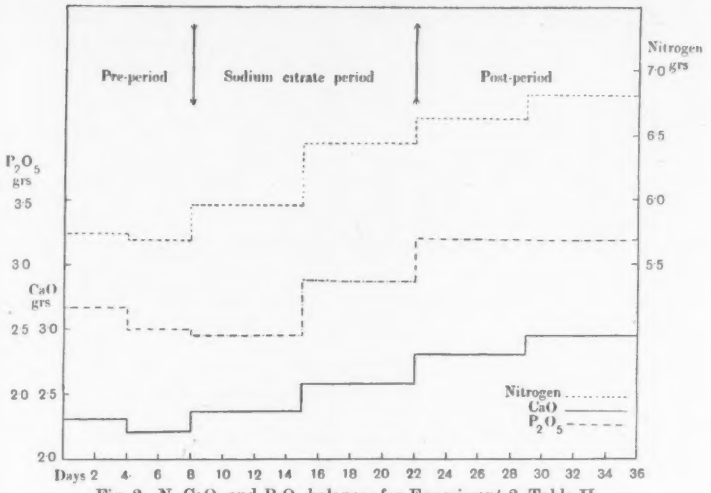

Fig. 2. N, CaO, and P_2O_5 balances for Experiment 2, Table II.

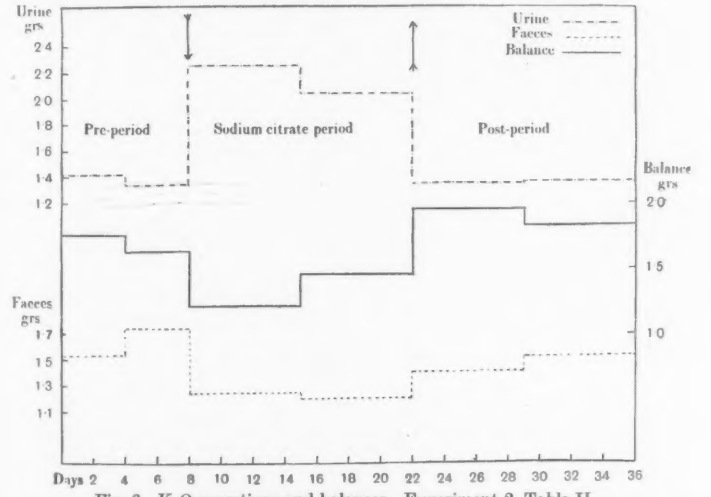

Fig. 3. K_2O excretions and balances. Experiment 2, Table II.

increased steadily during the experimental period to about twice the original amount, and in the post-period fell steadily back again. The following figures give the urinary excretion of CaO in grams per successive two day periods:

Pre-period	—	—	—	0·70	0·85	0·87	0·90
Sodium citrate period	0·76	0·85	0·97	1·30	1·31	1·36	1·43
Post-period	1·52	1·44	1·12	0·98	1·05	0·95	0·83

Taking the increased urinary excretion in conjunction with the increased CaO balance, this seems to point clearly to increased assimilation of CaO consequent on the addition of sodium salts to the diet.

Chlorine balance. Throughout both the experiments the chlorine balance remained negative. The addition of the sodium citrate seemed to have no lasting effect, a small positive balance for the first two days of the experimental period being counterbalanced by a large negative balance on the two following days. The average balance for the periods remained practically the same during the whole experiment. In Exp. 1, the addition of sodium chloride brought the animal nearly to equilibrium as regards chlorine. Of the additional chlorine fed practically the whole amount was excreted in the urine, the faecal excretion increasing only very slightly. On the other hand, the faecal excretion of Na_2O increased quite noticeably during the sodium chloride period, though the urinary increase was of course much greater. This would indicate that, of the additional sodium chloride fed, the animal assimilated more of the chlorine than of the sodium, in accordance with the fact that the chlorine balance was negative whereas that for sodium was positive.

Na_2O balance. In Exp. 1 the balance increased during the experimental period, and in the post-period went back to slightly less than in the pre-period. In Exp. 2 the large increase in the Na_2O balance for the first week of the sodium citrate period almost disappeared during the second week. Further, this positive balance was neutralised by a large negative balance which followed the withdrawal of the sodium citrate and was itself also only temporary.

K_2O balance. One of the most important points brought out by these experiments, and shown very clearly in Figs. 1 and 3, is to be found in a consideration of the K_2O results. Attention has already been drawn to the fact that in Exp. 1 the increased urinary excretion of K_2O was compensated for by the diminished faecal excretion (see Fig. 1); Exp. 2 confirmed this result. The excessive excretion of K_2O in the urine was largely counterbalanced by diminished excretion of K_2O in the faeces, and though the balance for the experimental period was below that of the pre-period, Fig. 3 shows that, for the second week, the balance was adjusting itself towards the former level, and withdrawal of the sodium citrate brought it immediately well above the original figure. From the analytical data we find that in Exp. 1 the total excretion of K_2O for the whole period of the experiment was 57·59 g. while the total excretion for the period, based on the average for the preliminary period, would have been 58·23 g. Taking the urinary excretion of K_2O, the amount actually excreted during the experiment was 17·98 g., while the amount based on the average of the preliminary period would have been 11·29 g. That is, while there was an increase of nearly 60 % in the urinary excretion of K_2O, the total amount of K_2O excreted by the organism was actually slightly less than normal. The figures for Exp. 2 show similar results. The total excretion of K_2O for the 36 days of the experiment was 110·59 g. as against 108·4 g. the amount calculated from the average of the preliminary period, *i.e.* the amount excreted was only 2 % above normal. The urinary excretion for the same period was 60·05 g. as against the calculated figure 49·54 g., *i.e.* the urinary excretion was 21 % above the normal.

This proves conclusively that urinary figures alone form no guide as to the balance of the organism in Na_2O and K_2O when the ratio of these is altered in the diet. Thus one of the points suggested by Bunge as necessary in future investigation, viz. analysis of the faecal excretion, turns out to be of such vital importance that quite erroneous conclusions have been drawn from the incomplete data of his experiments. Another of the points he brought forward, viz. the effect of continued administration of potassium, has been investigated by Miller [1923], who found in his experiments that the excessive excretion of sodium and chlorine in the urine was only transitory, little effect being observed after the first twenty-four hours period, even on continuing the doses of the potassium salt in the diet. This is not in accordance with our findings in the converse experiment. While there are indications in the figures for the experimental period that the animal is gradually adjusting itself to the excess of sodium salt in the ration, nevertheless after 14 days' feeding of sodium citrate, the amount of K_2O in the urine remains very much higher than in the pre-period, and withdrawal of the sodium salt is followed by an immediate drop in the urinary potassium excretion. Thus the effect in our investigation is by no means so transitory as Miller has found in his potassium feeding experiments.

It remains, therefore, still a moot point whether the organism would adjust itself so completely to the change of diet that the urinary excretion of K_2O would return to the original figure, but in view of our findings this point seems to us of minor importance. Of much more significance is the fact that the organism has apparently the power of adjusting the balance so that, even while abnormal urinary excretion continues, the balance may be very little disturbed, and when disturbed has the tendency to come back to normal.

The importance of a proper physiological balance between the mineral constituents of a ration has already been emphasised by Orr [1922, 1923], and further investigation is proceeding along these lines with special reference to the sodium-potassium ratio.

Conclusions.

1. The addition of sodium chloride or sodium citrate to a ration of cereal grain fed to a growing pig led to increased assimilation and retention of nitrogen, calcium and phosphorus.

2. The increase of sodium salts in the ration caused increased excretion of potassium in the urine, the increase being still very marked even after 14 days' continued administration of the sodium salt.

3. The excessive urinary excretion of potassium was more or less completely counterbalanced by diminished faecal excretion, with the result that the balance showed very little deviation from the normal, even during a period of very abnormal urinary excretion. Hence metabolic experiments must include analyses of both faeces and urine, otherwise the conclusions drawn may be completely erroneous.

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LXXXVII. THE METHYLATION OF THE CEREBROSIDES OF OX BRAIN.

(PRELIMINARY NOTE.)

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A series of investigations has been commenced in this laboratory in which it is hoped to apply to substances of biochemical interest, containing a sugar or similar residue, the methylation reaction which in the hands of Irvine [1923] has yielded results of the greatest importance in elucidating the structure of the simple and complex carbohydrates.

The present note deals with some preliminary results which have been obtained in a study of the cerebrosides phrenosin and kerasin, first isolated and studied in 1874 by Thudichum [1901], and later investigated notably by Thierfelder [1890], Rosenheim [1913], and Levene [1913]. From the results of these and other authors, it is clear that the two cerebrosides mentioned are galactosides in which the non-sugar residue consists of a complex formed from the base sphingosine ($C_{17}H_{35}O_2N$), and the fatty acids, phrenosinic acid ($C_{25}H_{50}O_3$) in the case of phrenosin, and lignoceric acid ($C_{24}H_{48}O_2$) in the case of kerasin. The constitutional formulae which accord best with their known properties are those of Rosenheim [1916]. So far no attention has been directed to the sugar residue of these compounds *as it occurs in situ* beyond its identification as galactose [Thierfelder, 1890]. Whilst all the naturally occurring glucosides so far examined have been shown [Irvine, 1923] to contain the stable 1 : 4 type of sugar, galactose is remarkable in view of the comparative ease with which it yields four isomeric pentacetates [Hudson, 1915; Hudson and Johnson, 1916] (divided into two pairs of ring isomers), and recently one of us [Pryde, 1923] has brought forward evidence in favour of the view that *normally* galactose does not possess the butylene oxide (1 : 4) internal linkage as hitherto assumed, but an amylene oxide bridge. The nature of the internal linkage of this hexose in naturally occurring galactosides such as the cerebrosides is therefore of interest in view of the possibility that it may differ from that of the free sugar, and that opportunities for testing this possibility are limited by the rarity of simple galactosides in nature. It is towards the solution of

this problem that the present investigation is mainly directed. The scheme of work embodies the complete substitution of the hydroxyl groups in the sugar residue by methoxyl, and the subsequent hydrolysis of the methylated complex by acid methyl alcohol. By this means the sugar fragment is obtained as a methylated methyl galactoside—since dry acid methyl alcohol brings about simultaneous hydrolysis and galactoside formation—in which the original position of the internal oxygen bridge is preserved unchanged.

The cerebrosides have been isolated from fresh ox brain using the pyridine method of Rosenheim [1914], and have been purified by repeated recrystallisation from alcohol-chloroform mixtures. As a preliminary to the main investigation, and in view of the fact that these compounds contain an unsaturated ethylenic linkage and a secondary nitrogen, it was necessary to discover if they could be submitted to the methylation reaction without degradation, and to establish conditions for this reaction and for the subsequent hydrolysis necessary for the isolation of the methylated fragments, more especially of the sugar residue. For this purpose small quantities of pure phrenosin and kerasin, originally prepared by Rosenheim, for which the authors beg to thank Dr J. A. Hewitt of King's College, London, have been subjected to methylation by means of dry silver oxide and methyl iodide.

Kerasin and phrenosin undergo methylation unchanged when submitted to the action of the agents mentioned above in a suitable solvent medium, six applications of the process being necessary to ensure complete substitution of the free hydroxyl groups. The initial stages of the methylation are tedious in view of the physical difficulties of solution, and the tendency of the partially methylated cerebrosides to separate during extraction as voluminous gels in the presence of such solvents as chloroform. As methylation proceeds these difficulties lessen and the compounds become more and more soluble in methyl iodide, rendering the use of extraneous solvents unnecessary. The results obtained in the case of kerasin are in agreement with the work of previous investigators, in that this cerebroside is found to take up five methyl residues to form pentamethylkerasin, which was obtained as a white, amorphous, pulverisable compound, melting at about 73°. In the case of phrenosin, however, methylation ceased at a stage intermediate between that required for a pentamethyl- and a hexamethyl-phrenosin, the product resembling pentamethylkerasin but having a lower melting point (35–40°). In view of the fact that two forms of this cerebroside have been reported, one containing one molecule of water less than the other, and if this loss of water involved the formation of an internal anhydride [Rosenheim, 1916], such a result might be anticipated if one were dealing with a mixture of the two forms. On the other hand this result is akin to the steric hindrance encountered in the methylation of the amyloses [Pringsheim and Persch, 1921, 1922]. The methylated phrenosin was, like the parent cerebroside, slightly dextrorotatory, having $[\alpha]^{16}_{5461}+7{\cdot}5°$ in methyl alcohol ($c = 0{\cdot}53$ %). The course of the two methylations is summarised in the following table:

Methylation of kerasin. Wt. used 1·47 g.

No. of methylation	Reagents	Extraneous solvent	Extractive solvent	Methoxyl content (%)	Melting point
1	20 cc. CH_3I 5 g. Ag_2O	30 cc. MeOH	EtOH : $CHCl_3$ 1 : 2	6·0	—
2	15 cc. CH_3I 4 g. Ag_2O	10 cc. MeOH	Hot EtOH	8·5	63°
3	7 cc. CH_3I 4 g. Ag_2O	—	Warm EtOH	13·15	45°
4	6 cc. CH_3I 3·5 g. Ag_2O	—	EtOH and a little MeOH	16·1	40–45°
5	6 cc. CH_3I 2 g. Ag_2O	—	EtOH	17·4	50°
6	5 cc. CH_3I 2 g. Ag_2O	—	EtOH	17·68	73°

Pentamethylkerasin requires 17·88 % OMe.

Methylation of phrenosin. Wt. used 1·1 g.

No. of methylation	Reagents	Extraneous solvent	Extractive solvent	Methoxyl content (%)	Melting point
1	7 cc. CH_3I 5 g. Ag_2O	5 cc. MeOH	Hot EtOH	3·6	—
2	7 cc. CH_3I 4 g. Ag_2O	3 cc. MeOH	„	5·8	75–80°
3	6 cc. CH_3I 4 g. Ag_2O	—	„	16·64	35–38°
4	6 cc. CH_3I 3·5 g. Ag_2O	—	„	18·27	35°
5	6 cc. CH_3I 2 g. Ag_2O	—	„	18·6	35°
6	6 cc. CH_3I 2 g. Ag_2O	—	„	18·75	35–40°

Hexamethylphrenosin requires 20·27 % OMe.

It is of interest to note that whilst the melting point of methylated kerasin falls and subsequently rises during methylation, such a rise was not observed in the case of phrenosin. This is in agreement with the suggestion already made that the latter contained an admixture of a second form.

The above preparation of pentamethylkerasin was subjected to hydrolysis by two methods: (1) the action of 2 % hydrochloric acid in dry methyl alcohol in a sealed tube at 100° for 20 hours; (2) boiling under reflux in 7 % sulphuric acid dissolved in dry methyl alcohol for 8 hours. In the latter case the alcoholic solution, after being neutralised with barium carbonate and filtered, deposited on cooling fine, white, feathery crystals, and oily globules which solidified on standing. The former consisted of the methyl ester of the fatty acid residue, and the latter of the sulphate of the base, but these were not further investigated. The methyl alcoholic solution was evaporated, more of the fatty acid ester was deposited, and finally, after removing the last traces of solvent at 100°/10 mm., the product was extracted with ether. The ethereal extract was evaporated, and after treatment in a vacuum at 100° gave a syrupy residue which was fractionally distilled in a high vacuum. A mobile colourless syrup distilled

over at 130°/1·0 mm., and was thus easily separated from the admixture of fatty acid ester which remained in the still. Working on this small test scale it was not possible to obtain more methylated sugar than sufficed for a determination of the refractive index (Abbé-Zeiss) and a small scale determination of the methoxyl content. The results found were n_D^{15} 1·4497 and OMe = 57·5 % and, whilst these are a little divergent from those required for a pure methyl pentamethylgalactoside (n_D^{13} 1·4484; OMe = 62·0 %), they clearly indicate that a separation of the methylated sugar residue is possible by the methods employed. It remains to carry these out on a scale capable of yielding sugar sufficient for its purification and for a full determination of its constitution.

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LXXXVIII. INSULIN FROM THE COD FISH.

THE DIRECT APPLICATION OF PICRIC ACID TO THE ISLET TISSUE.

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(*Received April 29th, 1924.*)

By the courtesy of Professor J. J. R. Macleod we were given advance information with regard to the interesting results, since published by McCormick and Noble [1924], of experiments on the insulin content of the principal islets which occur in the cod, halibut and other common fishes. This possible source of insulin was consequently explored in connection with the fisheries of this country, and although there is no clear prospect of its assuming practical importance in the commercial supply of the hormone in this country, the results obtained are worth recording.

The Toronto workers have shown that there is a large principal islet in the cod occurring as a cap over the top of the gall bladder. This principal islet is very easily detached and the method of preservation adopted by McCormick and Noble is to drop the islets, as soon as they are collected, into 70 % alcohol, containing about 0·3 % hydrochloric acid. Their results lead them to suspect that the amount of insulin in tissue so preserved diminishes according to the time elapsing before the material is worked up, and an experiment made in this laboratory suggests the same thing.

In considering alternative methods of collecting the tissue it appeared probable that use might be made of the facts that insulin is precipitated by picric acid and that the picrate obtained is soluble in watery alcohol and watery acetone. These properties were observed in previous work on the general nature of insulin, and the solubility of the picrate in mixtures of water and alcohol was mentioned in a recent paper published in conjunction with Starling [1924]. Dodds and Dickens independently discovered the solubility of the picrate in watery acetone and made use of this property in extracting insulin from mammalian pancreas [1924, 1].

Accordingly the following experiments were arranged.

Four 8 oz. medicine bottles were taken; in the first were placed 6 oz. saturated aqueous picric acid solution, in the second 4 oz. 5 % picric acid in absolute alcohol, in the third 4 oz. 5 % picric acid in acetone, in the fourth 6 oz. 0·3 % hydrochloric acid in absolute alcohol. These were sent on January

15th to Dr E. S. Russell, of the Ministry of Agriculture and Fisheries, with the request that islet tissue from freshly caught cod-fish should be added to each until the contents of the bottle reached the 8 oz. mark.

The tissue would thus be immediately fixed by picric acid in the case of the aqueous solution, and the insulin precipitated *in situ*, and it was thought possible that in bottles 3 and 4 the insulin might be converted into picrate and this automatically extracted by the approximately 60 % alcohol-water and acetone-water mixtures respectively, which would be obtained when 4 oz. islet tissue had been added to each bottle.

The fourth bottle was to be used for an extraction according to the method of McCormick and Noble.

Mr Michael Graham, Assistant Naturalist on the staff of the Ministry of Agriculture and Fisheries, spent a week at sea on a trawler in the early part of February, collecting islet tissue from absolutely fresh fish, but unfortunately the weather was very severe and the amounts of tissue which he was able to secure were so small that they could not be worked up satisfactorily.

A second similar series of collecting bottles was therefore supplied to him, and cod islets from the Yorkshire shore fisheries were collected. This material was not so fresh as that collected on the trawler, varying periods, up to 24 hours, elapsing between the catching of the fish and the removal of the islet tissue, but, nevertheless, relatively large amounts of insulin were found in it.

The material was collected on Feb. 21st, 26th, and 27th, 1924, and was worked up on March 5th.

The collection in alcoholic and acetone solutions of picric acid was only partially successful. Its proper trial involved a certain proportion between islet tissue and fixing fluid, which could not be observed. The preservation by McCormick and Noble's method also resulted in a very poor yield. It is probable that the tissue was not sufficiently fresh for success by this method. It is the more significant that, even with material of less than perfect freshness, preservation in watery picric acid was successful. Only the method of working up the material collected in this manner will be described in detail.

Extraction of insulin from islet tissue fixed in aqueous picric acid.

The aqueous picric acid solution was poured off and the actual pieces of tissue, which varied considerably in size, were counted and then weighed in the moist state. 108, corresponding to the same number of fish, weighed 9·6 g. They were then cut up with scissors and the tissue appeared to be stained uniformly throughout with picric acid. It was ground in a mortar with sand and returned to the picric acid solution. After standing for a day it was filtered off and the picric acid solution was discarded. The tissue was then ground repeatedly in a mortar with small amounts of 75 % acetone, each extract being filtered off. In all 150 cc. of the watery acetone were used. The combined

filtrates were evaporated *in vacuo*. After the acetone had been removed the picrate, with some fatty material, separated out, adhering mostly to the sides of the flask. The precipitate suspended in the watery layer was collected by centrifuging. It was then dissolved in about 5 cc. of a mixture of 75 parts of absolute alcohol and 25 parts of 3 *N* aqueous HCl. The solution was transferred to the flask and the remainder of the picrate and fatty material dissolved in it. The solution, which was rather turbid, was transferred to a centrifuge tube and the flask washed out with a further 5 cc. of the alcoholic HCl. The solution was centrifuged to remove the suspended matter and was then poured into 200 cc. acetone. The hydrochloride thus precipitated was filtered off, washed with acetone and ether and dried in the usual way [Dudley and Starling, 1924]. 0·126 g. hydrochloride was obtained. Its activity, expressed in terms of the original Toronto rabbit unit, proved to be 1 mg. per rabbit unit. Hence we obtained a yield of 1·17 rabbit unit per fish and 13·12 (or practically 40 clinical units) per gram of wet tissue. This yield is slightly greater than the highest recorded by McCormick and Noble, who in one instance got 35 clinical units per gram of islets from the pollack. The insulin content of this islet tissue is therefore of the order of ten times that of mammalian pancreas. It seems likely that the application of this same method of preservation would be even more successful if the islet tissue could be fixed while still perfectly fresh.

While the collection of our material was in progress we heard from Drs Dodds and Dickens of a process, which they had independently worked out for the preparation of insulin from mammalian pancreas and which involved the purification of a picrate, obtained from an original watery extract, by solution of the insulin-containing fraction in wet acetone [1924, 1]. We informed them of the above method, which we already had under trial for fish insulin, in which picric acid was applied directly to the original tissue. They have now tried an essentially similar process for extracting insulin from the mammalian pancreas, and have obtained very good yields [1924, 2]. It is possible that this method will prove applicable to the commercial production of insulin from slaughter-house material, but in any case we believe that the collection of fish islets in aqueous picric acid, and subsequent extraction of the insulin by the above method, will be found to have advantages wherever the circumstances make this source of insulin worth exploitation. A watery solution of picric acid can be transmitted to the site of collection without raising excise difficulties. It appears further, that, when once the fresh islet tissue has been dropped into an adequate quantity of this fixative, it can be kept for any time determined by convenience, transmitted without refrigeration to the factory where the extraction of the insulin is to be carried out, and still give a yield at least as good as that obtained with the greatest precautions as to speed and temperature, when the alcohol process of preservation is used.

Our best thanks are due to Dr E. S. Russell for his assistance in this investigation and particularly to Mr Graham for his careful collection of material under extremely trying weather conditions. I am greatly indebted to Dr H. H. Dale for very helpful suggestions in connection with this work and to Mr H. P. Marks for carrying out the physiological tests.

Summary.

(1) A convenient method of extracting insulin from the islet tissue of fish is described. It consists in applying picric acid directly to the tissue, extracting the insulin-containing picrate fraction with watery acetone, and converting the picrate into a soluble hydrochloride as described in a previous paper.

(2) From the islet tissue of the cod, although collected from fish which had been caught at varying intervals up to 24 hours previously, a yield of 13·12 rabbit units per gram of tissue was obtained. Probably more would be obtained from absolutely fresh islets. The islet tissue of the cod contains, weight for weight, apparently about ten times as much insulin as mammalian pancreas.

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LXXXIX. THE HYDROLYSIS OF PROTEIN BY A HEAT-STABLE CATALYST PRESENT IN MUSCLE.

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In a previous publication [Clifford, 1923] a rise of amino-nitrogen was recorded in meat extract and histidine solution, kept constantly at 95–100°, when cod muscle was present, whilst a simultaneous drop in iminazole content occurred. The amino-N rise might have been a simple heat hydrolysis of protein from the added tissue, or like the iminazole fall, the result of a catalytic action. Experiments were therefore carried out with protein solutions kept at 95–100° for various periods of time, and the rise of amino-N estimated by the method of van Slyke. Iminazole estimations [Clifford, 1921, 1] were also performed on the solutions.

Effect of Heat on Gelatin Solution.

A 5 % solution of gelatin was made with distilled water, and 10 cc. of this placed in each of several test tubes. The tubes were then plugged with cotton wool, and left in a constant boiling water-bath for 28–41 days. Water lost by evaporation was replaced each day. When required, a tube was removed, its contents made up to 95 cc. with distilled water, unaltered protein removed by the addition of 5 cc. of 20 % meta-phosphoric acid, and the filtrate used for amino-acid estimations, using van Slyke's micro-apparatus. From the remainder of the filtrate the iminazole content was determined according to the method previously described [Clifford, 1921, 1].

The results are shown in the figure, curves 43 and 54. A slight but definite rise of amino-N occurs, being 27·0 mg. per 100 cc. of gelatin solution in 28 days in Exp. 43 and 30·71 mg. in 41 days in Exp. 54.

The free iminazole content calculated as histidine, in Exp. 54, rose from zero on the first day of the experiment to 4·0 mg. per 100 cc. of solution on the 41st day. This probably was due to histidine liberated during the hydrolysis of the gelatin.

Effect of Heat on Albumin Solution.

A 5 % solution of dried egg albumin in distilled water was treated in a similar manner to that described for gelatin. Again a rise of amino-N took place, though considerably less than with gelatin. The results are shown in curves 47 and 53.

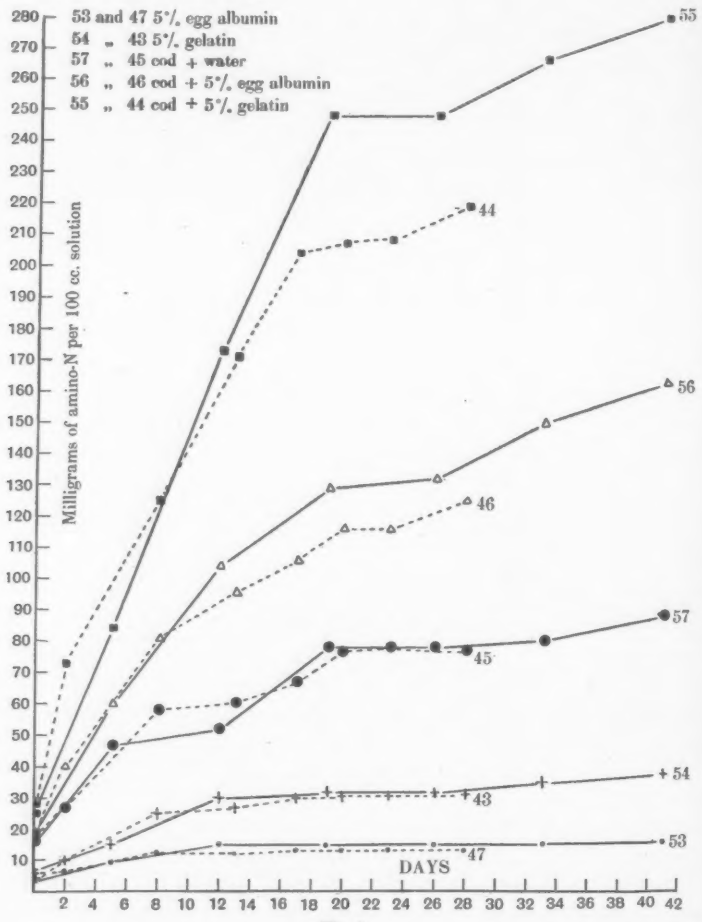

Fig. 1.

From these curves it can be seen that in 47 the rise of amino-N was 7·1 mg. per 100 cc. in 28 days, and in 53 was 13·5 mg. in 41 days.

The iminazole content, calculated as histidine, in 53 rose from zero to 6·0 mg. per 100 cc. in the 41 days. In these experiments the hydrolysis was

less than with gelatin, probably owing to the solubility of the latter, whilst the albumin was a firm solid mass at 95–100°.

Neither of these results is large enough to account for the rise of amino-N in iminazole solutions with muscle described in a previous paper [Clifford, 1923], where 10 cc. of 0·05 % histidine with 5 g. of cod kept in a constant boiling water-bath showed an increase of 43 mg. of nitrogen per 100 cc. in 21 days, and with 5 g. of washed beef showed an increase of 126 mg. in the same time. It seemed from these results that the increase of amino-N must either have been due to the actual structure of the muscle protein which lent itself more readily to hydrolysis than did egg albumin or gelatin, or else due to a catalytic action exercised by some substance in the muscle which accelerated protein hydrolysis at 95–100°.

Further experiments were carried out to determine these points.

Effect of Heat on Cod Muscle in Water.

Because cod muscle contains no extractable iminazole derivatives but at the same time possesses a heat-stable catalyst which destroys iminazole rings and apparently also a catalyst capable of hydrolysing protein, it was chosen as a source of catalyst.

The experiments were carried out as follows:

5 g. of minced cod muscle were placed in each of several test tubes, and about 10 cc. of water were added. The tubes were then plugged with cotton wool and treated exactly as described for gelatin.

Again there was a rise of amino-N, this time considerably greater than for either of the two simple protein solutions. The results are shown in curves 45 and 57.

From these results it appeared that simple hydrolysis could not account for the whole of the nitrogen rise, for since the protein of heated cod muscle more closely resembles coagulated albumin, in its solubility, than a gelatin solution, it would have been expected to show a hydrolysis similar to that of albumin in being less than that for gelatin.

There was a possibility that the extractives from the cod muscle had been split by a catalyst in the muscle as previously shown to be the case with the iminazole ring [Clifford, 1922], and the extra nitrogen derived in this way, or there might have been an acceleration of protein hydrolysis. It was also possible that both of these actions took place simultaneously. No diazotisable iminazole was found in the protein-free filtrate during the experiment, but a yellow colour was obtained similar to that previously found with cod muscle extracts [Clifford, 1921, 2].

Experiments were carried out to determine the effect of cod muscle on the hydrolysis of added protein. For this purpose gelatin and egg albumin were used.

Effect of Cod Muscle on Heated Gelatin Solution.

5 g. of minced cod muscle were placed in 10 cc. of 5 % gelatin solution in each of several test tubes, and these were treated as described for the former experiments. The rise of amino-N which occurred was found to be greater than the sum of the results obtained for gelatin alone and for cod and water alone. The results are given in curves 44 and 55.

Experiment 44 showed a rise of 197·3 mg. of amino-N per 100 cc. of solution in 28 days; and Exp. 55 a rise of 255·4 mg. in 41 days.

Thus there was an excess of 54·15 mg. in one case, and of 152·19 mg. in the other, over the nitrogen liberated from gelatin and from cod separately in a similar period of time. Apparently there is a heat-stable catalyst in cod muscle which can hydrolyse gelatin.

There was an absence of free iminazole in the protein-free filtrate all through the experiment as opposed to the slight rise in the case of gelatin alone. This was expected from earlier observations on the destruction of carnosine and histidine by cod muscle at 100° [Clifford, 1922, 1923]. The observations were then extended to egg albumin.

Effect of Cod Muscle on Heated Egg Albumin.

Experiments were carried out substituting 5 % egg albumin for the 5 % gelatin, and again adding 5 g. of minced cod muscle to every 10 cc. of protein solution.

A rise of amino-N greater than those from the two substances kept separately again occurred, but it was not so marked as with gelatin. The results are shown in curves 46 and 56.

In 46 there was a rise of 107·7 mg. per 100 cc. of solution in 28 days, and in 56 a rise of 134·2 mg. in 41 days. The excess of amino-N over the sum of the results from albumin alone and cod alone in these two experiments was 44·45 mg. and 48·2 mg. per 100 cc. solution respectively. The cod muscle catalyst is therefore capable of hydrolysing egg albumin.

Again there was no diazotisable iminazole all through the experiment (56), any liberated histidine being destroyed by the catalytic action of the cod muscle.

Discussion of Results.

There is a small but definite hydrolysis of protein, when a simple watery solution of gelatin or egg albumin is kept at 95–100° for 28–41 days.

This rise of amino-N is greater, when, in place of gelatin or egg albumin, cod muscle is substituted. In order to account for the increase, the muscle must be supposed to contain a substance capable of splitting protein or other nitrogenous matter present in muscle, with the liberation of free amino-groups.

When cod muscle is added to gelatin or to egg albumin solution, the rise of amino-N is considerably greater than that obtained by adding the results

of protein solution alone, to that of cod and water alone. Since in the cod and water, and in the cod and protein experiments there was an equal amount of cod muscle and therefore of extractives, it is reasonable to suppose that the increase of amino-N must have come from the hydrolysis of the extra protein in the gelatin and albumin tubes.

The fact that with albumin the rise of nitrogen is less than with gelatin can probably be accounted for by the insolubility of egg albumin at 100° while at that temperature gelatin is soluble, and more easily attacked by catalysts.

Free histidine naturally appears in the protein-free filtrate after the simple heat hydrolysis of protein. If cod be present, this iminazole is destroyed by the catalytic action of the muscle. The yellow colour obtained in the place of the red of diazotised histidine deepens throughout the experiment.

From these results it seems that there is a heat-stable catalyst in cod muscle which can hydrolyse protein, and gives a curve of the same type as that obtained for the destruction of iminazole if inverted [Clifford, 1922, 1923].

Experiments are in progress with the object of isolating this catalyst and freeing it from muscle proteins when it should be possible to decide whether the catalyst which hydrolyses proteins is identical with that which destroys the iminazole ring.

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Thanks are due to Professor V. H. Mottram for his interest and helpful criticism.

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XC. THE ANTISCORBUTIC VALUE OF FRESH AND CANNED ENGLISH TOMATOES.

By ELLEN MARION DELF.

(*Received May 1st, 1924.*)

The juice of raw tomatoes is known to be powerfully antiscorbutic, and according to American observers it possesses also both the fat-soluble A and the water-soluble B vitamins [Hess, 1920]. According to Hess and Unger [1918], *canned* tomatoes also have an important antiscurvy value even after a year's storage, and Hess states that the juice of canned tomatoes may be safely given as an antiscorbutic to young infants [Hess, 1920, p. 234]. Canned tomatoes have recently been introduced into the ration of the United States army "in lieu of an equal quantity of potatoes, not exceeding 20 % of the total issue" [Hess, 1920, p. 231]. The relative antiscurvy value of raw tomatoes appears not to have been determined, but Hess and Unger fed to young guinea pigs, on an otherwise scorbutic diet, the juice of tomatoes which had been canned and stored for a year. The minimal daily ration necessary to ensure protection from scurvy was found to be 4 cc. It is of some interest to speculate how this value would compare with that of the fresh juice.

In 1920, a number of experiments were started at the Lister Institute to determine this point for the English tomato. For various reasons, the publication of the results was delayed. Some canned tomatoes were however stored at the laboratory temperature and these have recently been tested for any residual content of vitamin C, after an interval of nearly four years. The results of these and of the former experiments are now presented in detail for the first time[1].

1. *Raw fresh tomatoes.*

These experiments were carried out from August to October 1919. The pulp and flesh of fresh English tomatoes was squeezed through muslin on alternate days and fed to young guinea pigs in daily doses. The remainder of their diet was crushed oats and bran and autoclaved milk, as in numerous other experiments conducted at the Lister Institute by various workers in connection with researches upon the distribution of the antiscurvy vitamin.

Groups of animals each receiving doses of 10 cc., 5 cc., and 2·5 cc. respectively were found to thrive well and exhibited no sign of scurvy at the post-mortem examination. Two animals (Nos. 575, 576), each receiving only 1·5 cc.

[1] The unpublished results have been quoted by the author [1921].

of the juice daily, remained also fairly active and their growth rate compared favourably with that of those receiving a higher ration, but both developed tenderness of the knee joints during the experiment and No. 575 became distinctly lame. Both these animals showed typical scorbutic lesions in the muscles and fascial tissues of the knees, and in both the tibiae were found to be distinctly brittle. The scurvy was however of a very mild character and in No. 576 it developed only during the last seven days of the experiment.

So far as these tests go, therefore, the minimum protective ration for a young guinea pig must lie between 1·5 and 2·5 cc. and may be estimated as about 2 cc. daily[1]. The juice is therefore not as efficient as orange or lemon juice. It is of interest to note that raw tomatoes have recently been tested in S. Africa and the local product is decidedly inferior to the English fruit, the minimum protective dose under similar experimental conditions being about 4 cc. daily[2].

2. *Bottled tomatoes.*

A sample of bottled tomatoes was received from Messrs Chivers in January 1919, which, according to information supplied at the time, had been prepared on Oct. 4, 1918. The fruit was first plunged into boiling water for two minutes to loosen the skin, then peeled and immediately packed into bottles without the addition of water. The bottles were "processed" by standing in a water-bath which was gradually heated to 190° F. (87·8° C.) and which was maintained for 45 minutes at that temperature.

This sample was tested by Miss M. Rhodes at the Lister Institute from Feb. to July 1919. The juice was therefore four months old at the beginning and nine months old at the end of the experiments. Two animals receiving 20 cc. and two others receiving daily 10 cc. of the juice from these tomatoes remained in good health for 90 days (Feb. to May). Two others receiving only 5 cc. daily (June, July) developed well-defined scurvy and were chloroformed after 42 days. No other tests were made. It should be noticed that the juice fed to the animals from June 12–27 was fermenting during the last days of use. The condition of the animals improved somewhat when a new supply had been obtained (June 27) but this sample also began to ferment on July 7; in spite of this it had to be used until July 15. It does not seem likely that fermentation alone was responsible for the somewhat severe symptoms observed and it may be concluded that the minimum protective dose for this sample of bottled tomatoes lies between 5 and 10 cc. It is thus considerably higher than the minimum dose of 4 cc. found by Hess and Unger for their American brand. If we take the minimum protective dose of the fresh juice as 2 cc., then about three-quarters of its value has been destroyed by the bottling and subsequent period of storage.

[1] In 1921 the minimum ration was quoted as 1·5 cc. The article was written in S. Africa, and at the time of writing the results of the post-mortem examination of Nos. 575, 576 had not been received.

[2] An account of these experiments by Delf and Pullinger is still in the press.

3. *Canned whole tomatoes.*

(*a*) *Before storage.* In August 1919, sound fresh English tomatoes were selected and canned in the following manner. After plunging into boiling water for two minutes, the tomatoes were peeled and heated gently for about ten minutes in a shallow pan, by which time juice had formed although no water had been added. Tins were filled with the fruit and juice and partly sealed. They were then processed at 100° C. for five minutes and finally sealed. The cans were cooled quickly by plunging into cold water and were stored at laboratory temperature. Some of these were at once tested for vitamin content in the usual way.

Two animals receiving 5 cc. of the juice daily developed severe scurvy; two receiving 7·5 cc. had good health throughout the experiment but developed a tenderness of the knee joints. At the post-mortem examination, this was seen to have been caused by slight subcutaneous haemorrhages. Two other animals receiving 10 cc. daily remained in perfect health, and at the post-mortem examination no trace of haemorrhage could be seen. The minimum dose for complete protection thus lies between 7·5 and 10 cc. daily, and is probably nearer 7·5 cc., instead of about 2 cc. as in the case of the raw juice. Mere canning has thus reduced the original value by about three-quarters.

(*b*) *After storage, from Aug. 1919 to May 1923.* The remaining cans were tested from May to August 1923 at University College, London by the courtesy of Prof. Drummond. The experiments were conducted as far as possible in the same way as those previously performed at the Lister Institute. Difficulties were encountered in the labour involved in feeding the rather large daily doses of the juice and the supply of the canned fruit came to an end somewhat prematurely, but some indication was obtained of the residual value of the juice after nearly four years' storage. The cost of these experiments was defrayed by a grant from the Food Investigation Board.

Three animals each receiving a daily ration of 7·5 cc. of the juice developed typical symptoms of scurvy during life, and when examined after death showed characteristic though not severe scorbutic lesions. In one case, haemorrhages around the knees were partly absorbed and this animal if kept longer would doubtless have become a case of typical chronic scurvy. Four animals were given a higher ration. Two of these had 10 cc. daily throughout the experiment. Of these, one soon developed severe scurvy and was killed after 35 days; the other showed no sign of scurvy and at the post-mortem examination, after 65 days, no haemorrhages were found, although the muscles were dark in colour. Two other animals received at first 2·5 cc. daily (for 16 days) and then 12·5 cc. daily (for 52 days), representing an average of 10 cc. each daily for the whole period. These both had good health, one never showing scorbutic symptoms and the other developing definite but not severe tenderness of the joints and a little lameness during the course of the experiment. At the post-mortem after 68 days of experiment, the former was found to have

a slight haemorrhage in the small intestine and the latter had two small haemorrhages in the intercostal muscles. These animals were therefore practically protected for 68 days, but showed signs of the onset of scurvy. For a longer time (*e.g.* 90 days as in the earlier experiments) probably 10 cc. would be insufficient but not much below the necessary minimum for protection. Storage has thus caused slow but progressive deterioration in vitamin content, the stored product having lost rather more than four-fifths of its original raw value as against a loss of about three-quarters of the original raw value when freshly canned.

4. Tomato purée canned.

In August 1919, ripe English tomatoes of good quality were made into a purée by being first heated slowly in an open pan until they just boiled (about 20 minutes) and then being strained through muslin. The pulp was then returned to an open pan and simmered at about 90° C. with frequent stirring until after one or two hours the resulting thick pulp weighed about one-third of the original fresh weight of the fruit, and occupied about half the original volume. This pulp was distributed into cans which were then sealed except for the vent, processed at 100° C. for five minutes and finally the vent was also sealed. The cans were then cooled quickly and tests were started the next day to determine the antiscurvy value of the resulting preparation, fresh cans being opened for the purpose every five days.

Very little protection from scurvy was given by a ration of 2·5 cc. of the purée daily, animals on this ration developing severe scurvy in 46 days. With a daily ration of 5 cc., active and healthy animals were produced. In one case on this ration, the only sign of scurvy was found in a few small haemorrhages in the intercostal muscles; in another, slight haemorrhages were found over the muscles of both knees and ribs although the general condition was good. A daily ration of 10 cc. gave perfect protection from scurvy. The minimal daily ration giving protection from scurvy in the case of this purée lies therefore between 5 and 10 cc. and may be estimated as about 7·5 cc. Allowing for the concentration during the preparation, this represents about 15 cc. of the fresh juice. It has been shown that 2 cc. of the raw juice would give protection and thus the double process of canning and concentration has so reduced the vitamin value, that from this point of view there is no longer any advantage in making the purée. By concentrating more rapidly in an atmosphere devoid of oxygen, the loss could no doubt be considerably reduced. The methods employed were those recommended at the time by the Canning Kitchen and are presumably in frequent use.

The foregoing results are summarised in the following table. They show conclusively that such routine examination of canned goods as is carried out at the present time does not give any criterion of the food value as far as vitamins are concerned [*cf.* Savage, 1923]. They also suggest that it would be a protection to the interests of the public, if the date of manufacture were

stamped on all canned goods, a course which has been frequently urged hitherto in vain. Storage probably always involves further slow deterioration in vitamin content even when it is claimed, that by use of chemical preservatives, the fruit can be kept for years with flavour unimpaired.

Table showing comparative antiscurvy value of fresh and preserved tomatoes.

	Minimal protective daily dose for guinea-pigs	Average
Pulp of fresh raw English tomato	1·5–2·5 cc.	2 cc.
Bottled tomatoes (Chivers), after 4–6 months' storage	5 cc.–10 cc.	7·5 cc.
Tomatoes canned whole, 1–2 months old	7·5–10 cc.	8·5 cc.
Tomatoes canned whole and stored 3¾–4 years ...	>10 cc.	—
Tomato purée canned	5–10 cc.	7·5 cc. purée (equivalent to 15 cc. original juice)

SUMMARY.

(1) The minimal daily ration of raw fresh juice of English tomatoes protecting young guinea pigs from scurvy has been shown to lie between 1·5 and 2·5 cc.; it is thus not quite as effective an antiscorbutic as fresh orange or lemon juice. Attention has been called to the inferior value of the locally produced S. African tomato.

(2) A sample of bottled tomatoes (supplied by Chivers) which had been stored for six months was found to have lost about two-thirds of its original value.

(3) By canning, a loss of nearly three-quarters of the original value was sustained. After storage for nearly four years, a further but smaller loss was found to have taken place.

(4) Canned tomato purée had lost about seven-eighths of the value of the original juice. The concentrated juice is bulk for bulk but little superior to the canned unconcentrated juice, when prepared as described in this communication.

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XCI. THE AMIDE NITROGEN OF CASEINOGEN.

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The work of Kühne [1877] and of many subsequent investigators has established the fact that the digestion of proteins with aqueous extracts of the pancreas, pancreatic juice, or commercial trypsin preparations fails to give complete hydrolysis. The observation of Fischer and Abderhalden [1903], that the proline and phenylalanine of caseinogen, haemoglobin, fibrin, egg albumin, and other proteins occur in a trypsin-stable complex, demonstrated in addition to the above a marked specificity in the action of trypsin. This conception of specific proteolysis was strengthened by later work of the same authors [1905] in an investigation of the action of trypsin on many synthetic peptides.

Very few observations have been made on the liberation of ammonia in the enzymic hydrolysis of proteins. The results of work on acid hydrolysis favour the assumption that the precursors of the ammonia are mainly amides of the dicarboxylic acids. The evidence on this point has been collected by Osborne [1924] and requires no repetition here. It need only be said that the general evidence is based on the rapid liberation of ammonia in the acid hydrolysis of proteins, on the absolute amounts of dicarboxylic acids and ammonia thus set free, and on the presence of asparagine and glutamine in the juices of plants.

Further light on the source of ammonia in the hydrolytic products of proteins is obtained from the following considerations:

(1) Maximum production of amino nitrogen is obtained by hydrolysis with 3 *N* hydrochloric acid for 1½ hours at 150°. Increase of temperature increases the ammonia at the expense of amino groups [Henriques and Gjaldbäk, 1910; Van Slyke, 1912].

(2) The additional ammonia obtained by prolonged acid hydrolysis at 150° does not arise from tryptophan [Andersen and Roed-Müller, 1915] or arginine, but partially from cystine [Henriques and Gjaldbäk, 1910].

(3) More ammonia is liberated by peptic digestion than by tryptic digestion. This is mainly due to the high acidity of the peptic digest [Henriques and Gjalbäk, 1911, 1912].

(4) Uramino acids are not hydrolysed by trypsin but are readily split by acid hydrolysis. Their presence in the products of enzymic hydrolysis of proteins has not been demonstrated [Andersen and Roed-Müller, 1915; Lippich, 1914].

(5) Levites [1904] could find no free $CONH_2$ groups in caseinogen.

(6) An imide linkage R-CO-NH-CO-R is improbable because of its resistance to acid hydrolysis.

Inasmuch as the ammonia nitrogen of proteins varies from 5 % to 30 % of the total nitrogen, it is obvious that ammonia is no mean constituent of the protein molecule and further evidence with respect to its association and behaviour is desirable. While engaged in some work requiring a digest of caseinogen, it was observed that prolonged hydrolysis of the protein with an aqueous extract of the pancreas failed to liberate the calculated amount of ammonia. It was therefore felt that a trypsin-stable residue might serve as a convenient material for investigation.

Part I.

In the experiments reported below, ammonia was estimated by rapid aeration for half an hour of a sample saturated with anhydrous potassium carbonate [Van Slyke and Cullen, 1914], Amino nitrogen estimations were made with the macro-apparatus of Van Slyke [1911, 1], and total nitrogen estimations by the Kjeldahl method. Clark and Lubs' buffers and indicators and the Cole-Onslow comparator were used in adjustments of the hydrogen ion concentration. Acid hydrolysis was effected by diluting if necessary to a convenient volume (25 cc. minimum) and refluxing for 16 hours with an equal volume of concentrated hydrochloric acid. The hydrolysate was evaporated to dryness *in vacuo* and subsequent determinations made upon the aqueous solution of the residue. If only the acid hydrolysable ammonia were to be determined it was found convenient to place in combustion tubes, $6'' \times 1''$, 5 cc. samples of suitable concentration and 0·5 cc. of concentrated sulphuric acid. Hydrolysis was then effected by autoclaving at 10 lbs. pressure. After two hours the tubes were removed. To each was added from a wide-mouthed pipette 5 cc. of a warm baryta solution (containing 2·95 g. of crystalline baryta, $Ba(OH)_2.8H_2O$, per 5 cc.) approximately to neutralise the sulphuric acid contained in the sample. The tubes were thoroughly cooled and the ammonia estimation proceeded with in the usual manner without transfer of the contents.

The enzyme solution used was a weak alcoholic (15 %) extract of fresh minced pig pancreas. Toluene alone was used as an antiseptic.

The liberation of ammonia in the tryptic hydrolysis of caseinogen.

The results of a typical experiment are given in Table I. Digestions were allowed to continue until the free ammonia content became constant and failed to increase after the addition of more enzyme solution.

It will be observed that the ratio Total ammonia/Total nitrogen is constant and in agreement with accepted values for the ammonia content of caseinogen, viz., 10·6 % to 10·7 % of the total nitrogen.

Table I.

300 g. caseinogen + 15 cc. toluene + 150 cc. enzyme solution + 20 g. sodium carbonate + water to 2 litres

Days		Decimilligrams nitrogen per cc. of filtrate: Total N	Free amino N	Free ammonia	Total ammonia	Amino N / Total N	Total NH_3 / Total N	Free NH_3 / Total NH_3	Adjustment to p_H 7·8. cc. added to whole digest
0·2	Volume made up to 2 litres	75	16·5	—	—	0·220	—	—	108·6 *N* soda
1	,,	131	—	—	—	—	—	—	123·0 ,,
2	,,	152	55·2	—	—	0·363	—	—	58·0 ,,
3	,,	138	55·9	—	—	0·405	—	—	24·1 ,,
5	,,	136	65·2	—	—	0·480	—	—	0·0 ,,
7	,,	133	67·0	—	—	0·503	—	—	4·2 *N* HCl
13	,, 100 cc. enzyme sol. volume made up to 2 litres	127	—	7·20	13·4	—	10·55	0·537	8·3 ,,
20		133	72·0	8·30	14·3	0·541	10·75	0·581	—
29	Volume made up to 2 litres 100 cc. enzyme solution	113	62·0	7·90	12·4	0·549	10·95	0·636	6·2 *N* HCl
40		105	59·0	7·50	11·1	0·561	10·55	0·675	—
44		106	59·3	7·50	11·3	0·559	10·65	0·663	—

The titration values are of some interest. Assuming that all the phosphorus in caseinogen (0·85 %) is present as orthophosphoric acid, 140 cc. of normal alkali would be required for complete neutralisation of the phosphoric acid in 300 g. of caseinogen. The sodium carbonate initially added is equal to 215 cc. of normal alkali. It would therefore appear that in the early stages of tryptic hydrolysis, the substances liberated were mainly neutral and acid ampholytes. In the later stages of hydrolysis, the tendency of the solution to become more alkaline than p_H 7·8 would seem to indicate a relatively great liberation of the hexone bases.

For the sake of brevity, only the final ratios Free ammonia/Total ammonia have been given in Table II.

Table II.

		Free NH_3 / Total NH_3
Digest 2	18 days	0·643
,, 3	12 ,,	0·658
,, 4	56 ,,	0·646
,, 5	50 ,,	0·667
,, 6	50 ,,	0·679
,, 1	44 ,,	0·663

While the time required for the completion of ammonia production is subject to much variation—the relative constancy of the final ratio is obvious.

Two possible causes of this failure to attain complete hydrolysis of the ammonia precursors might be presented: first, depression of the point of equilibrium by the accumulating end products of tryptic hydrolysis and inactivation of the enzyme, or secondly, the liberation of a trypsin-stable residue. The first possibility is suggested by the work of Abderhalden and Gigon [1907]. In studying the effect of many amino acids upon the cleavage of *d*-alanyl-

d-alanine and glycyl-*l*-tyrosine by pressed yeast juice, they observed marked inhibition by *dl*-leucine, *dl*-valine, and *d*-glutaminic acid—particularly the last. Northrop [1921] in a series of investigations on the inactivation of trypsin, observed the time required to effect a fixed change in the conductivity of a gelatin-trypsin solution by:

(1) trypsin-hydrolysed gelatin and caseinogen;
(2) acid-hydrolysed caseinogen;
(3) alkali-hydrolysed caseinogen.

No inhibition was observed with the totally hydrolysed protein (2) and (3). Positive results were obtained with the trypsin-hydrolysed products.

The second possibility has already been referred to on page 679.

The results recorded in Table III indicate that the failure of trypsin completely to hydrolyse the ammonia precursors in caseinogen, is due not to inactivation of the enzyme or depression of the point of equilibrium, but to the specific nature of the substrate. The tryptic digestion of preparations 9 and 10, each of 1200 g. caseinogen was allowed to continue until the free ammonia content failed to be increased by the addition of fresh quantities of enzyme solution. The digests were then filtered, neutralised to p_H 7·0 and concentrated *in vacuo* at 35°–40° to a volume of 1200 cc. The amino acids of lower solubility which separated out during concentration were filtered off. The final syrup was warmed to 60° and precipitated with four volumes of warm 95 % alcohol. The deeply pigmented oil which immediately settled out was run off through a separating funnel. By this procedure, about one half of the trypsin-stable residue was precipitated. This was dissolved in water and reconcentrated *in vacuo* for the removal of alcohol. The concentrate was finally taken up in water, diluted to five litres, adjusted to p_H 7·8 and redigested with an activated aqueous extract of the pancreas. As is shown in Table III, no increase in the free ammonia content, and hence no further hydrolysis of the ammonia precursor took place.

Table III. *Redigestion of the alcoholic precipitate.*

	Free ammonia	
Days	Digest 9	Digest 10
0	2·30	3·65
1	2·35	3·60
3	2·37	3·65
5	Fresh enzyme added	Fresh enzyme added
6	—	3·60
7	—	3·70
10	2·35	—

It is therefore concluded that in the tryptic digestion of caseinogen a portion of the ammonia representing approximately one-third of that which may be obtained by total acid hydrolysis, fails to be liberated by the enzyme. The residue isolated after digestion totally resists tryptic hydrolysis (of the ammonia precursor) and in consequence is considered to be structurally different from the trypsin-hydrolysed portion.

Part II.

Isolation Experiments.

The results of many preliminary isolation experiments may be briefly summarised.

1. Phosphotungstic acid quantitatively precipitated the trypsin-stable precursor of the ammonia. The simultaneous precipitation of the hexone bases and the acidity required for its use, were serious limitations. The proline-phenylalanine compound of Fischer and Abderhalden [1903] and possibly other undesirable peptides were also precipitated by this reagent.

2. Alcoholic mercuric chloride gave quantitative precipitation at p_H 7·0 of the ammonia precursor, but the increasing acidity during subsequent treatment with hydrogen sulphide was a source of trouble. The simultaneous precipitation of the hexone bases was also an undesirable feature.

3. Prolonged fractional precipitation with both aqueous and alcoholic mercuric chloride gave no separation.

4. The combined use of lead acetate and alcohol [Mack, 1904] failed to give quantitative precipitation and effected little additional purification if any (see Table V).

5. The known low solubility of copper aspartate and glutaminate and the great solubility of the copper salts of polypeptides [Fischer, 1906], led to the use of barium hydroxide [see Kober and Sugiura, 1913], and alcohol as precipitants of the copper salts. The results indicated that no free dicarboxylic acids were contained in the material that had been first precipitated by lead acetate and alcohol. Methods 4 and 5 were each capable of giving a yield of only 30 % to 50 % of the calculated amount. In consequence the use of these in sequence involved a great loss of material.

It was however found that three to seven volumes of 95 % ethyl alcohol would precipitate 50 % to 70 % of the trypsin-stable ammonia precursor. The advantages of its use are that the precipitant may be readily removed, that by precipitating at p_H 6·0–7·0, the free monoamino-monocarboxylic acids (all of which are undissociated in this range) are not precipitated, and that by precipitating with warm alcohol, the precipitate at once separates as a deeply pigmented oil which may be readily extracted at different acidities with ethyl alcohol of various concentrations.

Alcoholic precipitation and extraction.

The redigested solutions from digests 9 and 10 (see p. 682) were neutralised. To each was added a 50 % solution of normal lead acetate until a precipitate ceased to form. This procedure undoubtedly precipitates a portion of the ammonia precursor, but is desirable for clearing the solution. The filtrates were treated with hydrogen sulphide for the removal of lead, filtered free of lead sulphide, and concentrated *in vacuo* for the removal of hydrogen sulphide.

The partially concentrated filtrates were neutralised with soda and again concentrated to a final volume of 1000 cc. Four volumes of warm 95 % alcohol were added. The precipitated syrups of 300–350 cc. were then extracted as follows:

1. With 90 % alcohol—after adding increasing amounts of sodium hydroxide solution.

2. At p_H 7·0 with ethyl alcohol of decreasing strength.

The initial precipitations at neutrality with ethyl alcohol will have given a material free from the monoamino monocarboxylic acids. By the first set of extractions, it was hoped that residual free amino acids, if any, with alcohol-soluble sodium salts (*e.g.* histidine) would be removed. In the second set of extractions, the removal of any free amino acids would be facilitated by the increasing dilution. This procedure is also effective in totally removing the free ammonia. The extractions were effected by rapid shaking in a mechanical shaker in the order shown. Acetic acid was used in acidifying from p_H 11·0 to 7·0, because of the solubility of sodium acetate in alcohol.

Table IV.

		Alcoholic extractive				Percent. of total N	
	Extraction time	Volume cc.	Conc. %	p_H of solution	Total N	Free amino N	Total amino N
1 a	15 minutes	150	90	7·0			
b	,,	,,	,,	,,			
c	,,	,,	,,	,,			
d	,,	,,	,,	,,			
2 a	15 minutes	150	90	9·0			
b	,,	,,	,,	,,			
c	,,	,,	,,	,,			
d	,,	,,	,,	,,			
3 a	15 minutes	150	90	11·0			
b	,,	,,	,,	,,			
c	,,	,,	,,	,,			
d	,,	,,	,,	,,			
4 a	15 minutes	150	95	7·0			
b	,,	,,	,,	,,	100	53·0	78·0
c	,,	,,	,,	,,			
d	,,	,,	,,	,,			
5 a	15 minutes	150	90	7·0			
b	,,	,,	,,	,,			
c	,,	,,	,,	,,			
d	,,	,,	,,	,,			
6 a	15 minutes	150	85	7·0			
b	,,	,,	,,	,,			
c	,,	,,	,,	,,			
d	,,	,,	,,	,,			
7 a	15 minutes	150	80	7·0			
b	,,	,,	,,	,,	100	54·3	79·3
c	,,	,,	,,	,,			
d	,,	,,	,,	,,			

In digest 10 the combined extracts 4 and 7 each of 600 cc. were concentrated to 100 cc. Total nitrogen, free amino nitrogen and total amino nitrogen were estimated as shown.

In Table V are given the results of nitrogen estimations on

1. The extraction product from digest 9.
2. The extraction product from digest 10.
3. The product obtained from digest 4 by precipitating with lead acetate and alcohol.
4. The material obtained by twice precipitating with alcohol the calcium salt of the extraction product from digest 9 and subsequently removing the calcium with oxalic acid.

Table V.

	Total N	Free amino N	Total amino N
1. Extraction product (9)	100	52·5	82·5
2. Extraction product (10)	100	53·3	80·9
3. Lead acetate with alcohol (4)	100	49·0	82·0
4. Calcium salt of (1)	100	50·9	82·5

The nitrogen estimations as given in Table IV and V indicated sufficient constancy in this product of prolonged tryptic digestion of caseinogen to justify a total analysis of the product.

All preparations gave a very marked biuret test.

Analysis. 103 g. of the clear syrupy extraction product 10, representing 40 grams of material dried at 110°, were dissolved in 225 cc. of water; 75 cc. of concentrated sulphuric acid were added. After 24 hours at 100°, the biuret test was negative and hydrolysis was considered to be complete. The solution was diluted with two volumes of water. A warm saturated solution of recrystallised baryta was added until distinctly alkaline to phenolphthalein. Ammonia was removed and estimated by vacuum distillation into standard acid. After two hours' distillation, the excess baryta was neutralised to p_H 7·0 by the addition of sulphuric acid. The barium sulphate was removed by filtration and three times extracted by shaking with one litre of boiling water. The combined neutral filtrates were concentrated to 100 cc.

Continuous extraction with butyl alcohol, as recommended by Dakin [1918], was used for removal of proline and the monoamino monocarboxylic acids. The extraction was effected under diminished pressure to inhibit anhydride formation. The butyl alcohol rapidly became light brown in colour but only a very slight separation of amino acids appeared on the flask at the butyl alcohol surface. The extraction was continued for 30 hours.

The extracted aqueous solution was concentrated *in vacuo* for the removal of butyl alcohol. The residual syrup was dissolved in water and diluted to 200 cc.

The hexone bases were determined by precipitation of a 10 cc. portion with phosphotungstic acid in the usual manner. The washed precipitate was decomposed by shaking with washed amyl alcohol and ether. The individual bases were estimated by the partition method of Van Slyke [1911, 2].

The 190 cc. residue was saturated at $-5°$ with dry hydrogen chloride. The glutaminic hydrochloride was filtered off on asbestos after 24 hours at 0° and washed with 75 cc. of cold concentrated hydrochloric acid. The filtrate and washings were concentrated *in vacuo* to 100 cc. and resaturated as above with hydrogen chloride. After 24 hours the second crop of glutaminic hydrochloride, which was very small in quantity, was filtered off. The combined hydrochloride fractions were dissolved in 90 cc. of water and purified by reprecipitation with hydrogen chloride. The hydrochloride was filtered off after 24 hours and washed with cold absolute alcohol and ether.

The combined filtrates from the glutaminic hydrochloride were evaporated *in vacuo* to a syrup. The residue was dissolved in water, and distilled *in vacuo* with an excess of pure calcium hydroxide. A very small amount of ammonia which escaped the previous alkaline distillation was caught in standard acid and estimated. The excess calcium hydroxide was filtered off and the filtrate and washings were concentrated to 100 cc. The calcium salts were precipitated with alcohol according to the method of Foreman [1914]. The precipitated calcium salts were dissolved in water. The calcium was exactly removed with oxalic acid.

Aspartic acid was separated as the lead salt [Dakin, 1918] and estimated as the copper salt. The solution free from calcium was heated on a water-bath with an excess of freshly precipitated and well washed lead hydroxide. After 15 hours the lead precipitate was filtered off and washed. It was decomposed by boiling with dilute sulphuric acid. Excess of the latter was removed with barium carbonate. The filtrate was concentrated and treated with hot saturated copper acetate. After a few days, deep blue aggregates of copper aspartate separated out.

The filtrate from the lead aspartate was treated with hydrogen sulphide for the removal of lead. The filtrate was freed from hydrogen sulphide, acidified to 5 % with sulphuric acid and precipitated with phosphotungstic acid. After 48 hours the voluminous precipitate was filtered off. Phosphotungstic and sulphuric acids were exactly removed from the colourless filtrate with baryta. Chlorides were removed with silver nitrate and nitric acid. The filtrate, free from chlorides, was made slightly alkaline to litmus with soda. The further addition of silver nitrate and soda until the completion of precipitation brought down a voluminous precipitate. This was suspended in water and treated with hydrogen sulphide for the removal of silver. The filtrate and washings from the silver sulphide were concentrated *in vacuo*. A few colourless crystals separated out after 24 hours. The residual solution of a few cc. was precipitated with alcohol and ether. The flocculent white precipitate was filtered off, dissolved in water and reprecipitated three times. The crystals and the ether precipitate melted at 198° and proved to be *dl*-glutaminic acid. No β-hydroxyglutaminic acid could be found.

Neither the butyl alcohol-soluble fraction nor the fraction extracted by, but insoluble in, butyl alcohol was in sufficient quantity for analysis.

	Grams	Percent.
Glutaminic acid	21·80	54·50
Lysine	7·30	18·25
Ammonia	0·50	1·25
Arginine	1·55	3·88
Aspartic acid	0·59	1·48
Butyl alcohol solution	0·55	1·38
Butyl alcohol extracted	0·05	0·12
	32·34	80·90

The above analysis shows that if all of the ammonia (0·50 g.) that has been liberated by acid hydrolysis is associated with a single amino acid, then only glutaminic acid and lysine can be considered as parts of the trypsin-stable ammonia precursor. The arginine content is one-fourth of the amount that would be required for the existence of even equimolecular amounts of arginine and ammonia. For the purposes of this investigation, it would therefore appear that the arginine and aspartic acid were impurities.

Evidence will now be presented that this fraction of the ammonia is not associated with the lysine. A portion of the final extraction product from digest 4 was precipitated with phosphotungstic acid, following a preliminary precipitation with alcoholic mercuric chloride. The phosphotungstic precipitate was decomposed by adding gradually a warm solution of baryta with vigorous shaking until the precipitation of barium phosphotungstate and barium sulphate was complete. These were removed by filtration. The residue was twice extracted with boiling water. The combined filtrates were concentrated *in vacuo* to 500 cc. and found to contain 3·34 g. of amino nitrogen. The solution was shaken for 6 hours with an ethereal solution (200 cc.) of 67·5 g. of β-naphthalenesulphonic chloride (25 % excess); 40 % soda was added at intervals of one hour to maintain the contents alkaline to phenolphthalein. A brown syrupy mass remained in the agitation bottle. The clear supernatant liquid was decanted into a separating funnel for the removal of the aqueous layer. The latter was acidified with hydrochloric acid and was placed in the ice chest over night. The white crystalline mass which separated out weighed 5·5 g. and was nitrogen-free. It was probably β-naphthalenesulphonic acid.

The oily residue which separated from the alkaline solution was taken up in 75 cc. of warm 95 % alcohol, and slowly poured with much stirring into 1000 cc. of cold dilute hydrochloric acid. A gummy mass separated and hardened into a granular mass. After three days the aqueous layer was decanted off and the powdered residue washed repeatedly with cold water. Ether extraction of a portion was attempted but was found impracticable owing to softening. The whole was then boiled for half an hour with 1500 cc. water and the hot portion was decanted off. The residue rapidly hardened to a brown transparent mass. Very little material appeared to be dissolved by the water. The dry residue was dissolved in 100 cc. of warm 95 % alcohol and poured slowly into two litres of cold ether. After 24 hours in the ice chest, the brown gummy residue was separated by decantation. On again treating

with alcohol, a small portion of about 1·5 g. failed to go into solution after repeated boiling. The portion readily soluble in 75 cc. alcohol was reprecipitated by slowly pouring into 2500 cc. of water at 0°. The white granular precipitate was dried by vacuum desiccation to constant weight; 8 g. were obtained containing 7·65 % of nitrogen. 5·85 g. of the material were boiled with 200 cc. of 1 : 1 hydrochloric acid for 7½ hours. The yellow supernatant liquid (Solution A) on cooling, was poured off from the brown insoluble portion. The latter was boiled with 100 cc. of water, and dissolved in alcohol. The alcoholic solution was concentrated to dryness by vacuum desiccation and gave a syrupy residue which rapidly hardened to a brittle, granular mass.

0·1022 g. after drying for five hours at 105° weighed 0·1004 g.

The total vacuum-dried product weighed 2·511 g. Duplicate carbon and nitrogen estimations were made.

	% C	% N
Found	59·0	5·28
Calculated for di-β-naphthalenesulphonic deriv. of lysine	59·3	5·33

The formation of the di-β-naphthalenesulphonic derivative of lysine is only possible when the amino groups do not occur in peptide linkage. We may therefore assume one of the following possibilities:

(1) That the lysine contained in the isolation product is free and not in peptide linkage through its carboxyl groups.

(2) That the carboxyl group of the lysine is in ordinary peptide linkage with another amino acid

$$NH_2—CH_2—CH_2—CH_2—CH_2—CH(NH_2)—CO—NH—CH...$$

(3) That the carboxyl group of the lysine is in an imide linkage

$$NH_2—CH_2—CH_2—CH_2—CH_2—CH(NH_2)—CO—NH—CO—R$$

(4) That the isolation product contains lysine amide. The first and second assumptions have no bearing on the lysine and ammonia relationship and may be disregarded. A compound of the third type is split by alkaline hydrolysis but is unchanged by boiling with mineral acids [Bergell and Feigl, 1908]. It would not be split by acid hydrolysis. The occurrence of lysine amide is not possible, for the following reasons:

(1) If the amide group were to react [Königs and Mylo, 1908] with β-naphthalenesulphonic chloride, a carboxy-sulphonimide compound would be formed which is resistant to acid hydrolysis, ...CONH—SO_2...

(2) If the amide group were not to react [Bergell, 1914] with β-naphthalenesulphonic chloride, subsequent acid hydrolysis would liberate ammonia in equimolecular amounts. The hydrolysis of 5·85 g. of the β-naphthalenesulphonic derivative produced 8 mg. of ammonia nitrogen in solution A in place of the 69 mg. required by theory.

Solution A containing approximately 3·3 g. (by difference) of the hydrolysed derivative was evaporated to a syrup and dissolved in 200 cc. of boiling water. On cooling the solution the material separated out as oily drops which failed

to crystallise (from the aqueous supernatant layer 100 mg. of glutaminic acid were obtained as the hydrochloride). The material was repeatedly precipitated from alcoholic solution by pouring slowly into a rapidly stirred freezing mixture of ice and concentrated hydrochloric acid, but no crystalline product could be obtained.

The largest amount of ammonia in protein hydrolysis has been found in certain of the vegetable proteins—the prolamines. The fact that the members of this group contain no lysine [Osborne and Clapp, 1907] may therefore be advanced in further support of the view that the ammonia of the trypsin-stable residue in caseinogen is not in direct association with the lysine.

It may now be shown that the ammonia of the trypsin-stable residue is present in amide form. The collected results of the work on amide hydrolysis by tissue enzymes *in vitro* [Gonnerman, 1902, 1903; Bergell and von Wülfing, 1910, 1, 2; Bergell and Brugsch, 1910; Schwarzschild, 1904; Lang, 1904] may be summarised as follows.

Of seventeen different amides and imides the pancreas hydrolysed three and gave negative results with fourteen; the liver hydrolysed thirteen and gave negative results with four, the kidney hydrolysed nine and gave negative results with six.

The work of Lang [1904] on deamidation has been referred to by many investigators. Previous workers, Bergell and v. Wülfing [1910, 1, 2] and Schwarzschild [1904], had reported that asparagine was not deamidised by pancreas extracts. Lang tried asparagine with preparations of the liver, kidney, testes, spleen, and intestinal mucosa. Glutamine was used in one experiment with a liver preparation only. Unfortunately these amides were not used with pancreas extracts. His conclusions were as follows: "Diese Amide (Asparagine and Glutamine) wurden in allen Organen, *denen sie zugesetzt wurden*, desamidiert." Subsequent references to Lang's conclusions have heightened the impression that all animal tissues deamidised these substances, whereas the pancreas would probably have given negative results had it been tried [See Bostock, 1912; Effront, 1917; Cathcart, 1921].

It seemed desirable to investigate the behaviour of the trypsin-stable residue with preparations of the pancreas, liver and kidney. Five g. of each of these organs from the pig were finely minced and ground with washed sand. The ground tissue was washed out with water from time to time until 50 cc. of the suspension were obtained. To the pancreas preparation were added 5 cc. of a similar extract of the mucosa of the small intestine.

To 6 cc. of a 5 per cent. solution of the isolated residue from digest 4 were added 2 cc. of the tissue suspension and 0·5 cc. of toluene. Duplicate experiments were made at initial p_H of 5·6, 6·2, 6·8, 7·4, 8·0, 8·6. The tubes were well-shaken, corked, and kept for four days at 40°.

	Nett cc. (experimental – blank) of 0·1 *N* ammonia					
p_H	5·6	6·2	6·8	7·4	8·0	8·6
Pancreas	0·0	0·0	0·0	0·0	0·0	0·0
Liver...	0·05	0·30	0·50	0·65	0·70	0·30
Kidney	0·05	0·30	0·30	0·35	0·35	0·05
Acid hydrolysis	—	—		1·60	—	—

This experiment again demonstrates the failure of pancreas extracts to effect deamidation of the trypsin-stable residue. It is also possible that the ammonia liberated by the liver and kidney preparations arose from deamination of α-amino groups.

The effect of liver and kidney preparations was then tried on:

(1) The extraction product from digest 9.

(2) The extraction product from digest 9 after hydrolysis with 20 % sulphuric.

(3) Gelatin hydrolysed by acid.

If the ammonia is a product of deamidation, positive results would be obtained only with the unhydrolysed material (1). If the mechanism is one of deamination—the α-amino group of (2) and (3) should be attacked.

The experiments were performed in duplicate at a single initial p_H of 7·4.

	cc. 0·1 *N* ammonia	Average
2 cc. liver (pig) suspension + 6 cc. water	0·23	0·23
6 cc. (9) hydrolysate	0·60 0·55	0·58
6 cc. (9) unhydrolysed	0·00	0·00
6 cc. gelatin hydrolysate	0·60 0·70	0·65
2 cc. liver + 6 cc. (9)	1·85 1·90	1·88
2 cc. liver + 6 cc. (9) hydrolysate	1·00 1·00	1·00
2 cc. liver + 6 cc. gelatin hydrolysate	1·30 1·30	1·30
2 cc. kidney + 6 cc. (9)	1·65 1·55	1·60
2 cc. kidney + 6 cc. (9) hydrolysate	0·80 0·80	0·80
2 cc. kidney + 6 cc. gelatin hydrolysate	0·85 0·90	0·88
2 cc. kidney + 6 cc. water	0·22	0·22
Acid hydrolysis of 6 cc. (9)	2·15	2·15

Recalculated	Gross	Blanks	Nett
Liver + (9)	1·88	0·23 0·0	1·65
„ + (9) hyd.	1·00	0·23 0·58	0·19
„ + gelatin...	1·30	0·23 0·65	0·42
Kidney + (9)	1·60	0·22 0·0	1·38
„ + (9) hyd.	0·80	0·22 0·58	0·00
„ + gelatin	0·88	0·22 0·65	0·01

While slight deamination has been effected by the liver, the results obtained with the kidney preparations indicate marked deamidation and no deamination. The production of ammonia from only the unhydrolysed isolation product is advanced as evidence in support of an amide structure for the ammonia precursor. This is again supported by the analogous behaviour of amides with liver, kidney, and pancreas preparations,—referred to on page 689.

SUMMARY.

1. In the prolonged tryptic digestion of caseinogen about one-third of the total ammonia that may be obtained by acid hydrolysis fails to be liberated by the enzyme.

2. The isolated residue totally resists tryptic hydrolysis of the ammonia precursor and is considered to be structurally different from the portion hydrolysed by trypsin.

3. About one-half to two-thirds of the trypsin-stable residue may be precipitated by ethyl alcohol. Total precipitation is obtained with phosphotungstic acid and alcoholic mercuric chloride.

4. The acid hydrolysate of the alcohol-precipitated and extracted residue consists almost entirely of glutaminic acid and lysine. Eight to ten per cent. of the total nitrogen occurs as ammonia.

5. It is shown that the ammonia contained in the trypsin-stable residue is not associated with the lysine.

6. The behaviour of the material with enzyme preparations from the pancreas, liver and kidney of the pig was investigated. The results confirm the view that the ammonia is present in amide form.

It may therefore be concluded that glutamine, or a glutamine-containing peptide, is present in the tryptic digestion product of caseinogen. While it is probable that the glutamine is an integral part of the caseinogen molecule,—the possibility of the secondary formation of the amide by an enzymic synthesis cannot be neglected.

The writer is indebted to Professor F. G. Hopkins for advice and supervision and to the Royal Commissioners for the Exhibition of 1851 for the studentship that has been held during the period of this investigation.

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XCII. INVESTIGATIONS INTO THE CIRCULATION OF SOME HEAVY METALS IN THE ORGANISM (MERCURY, BISMUTH AND LEAD).

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The investigations contained in this paper comprise a series of quantitative analyses, performed with the aid of two very sensitive analytical methods. These methods we owe, the one (Hg), to Dr Christiansen, the other (Bi and Pb), to Prof. G. Hevesy.

The three metals in question show a great similarity in the organism, which makes it reasonable to subject them to a comparative examination.

The experiments were almost all made on rabbits, and carried out in the following way. The rabbit is placed in a cage which permits of the collecting of all the urine and faeces eliminated daily. The metal compound to be investigated is injected at short intervals, in different portions of the muscles of the animal's limbs. After 10–20 days the rabbit is killed under a narcotic by bleeding. The principal organs, heart, lungs, liver, kidneys and intestines, together with a sample of the blood, are removed for examination.

After this the portions of the muscle, where the injection has been performed, are excised in order to ascertain the amount of metal remaining. In some cases it was comparatively easy to find and excise the parts where the injection has taken place, which contain the unabsorbed remains of the metal. In other cases it was considerably more difficult. With bismuth hydroxide it was even necessary to add a little lamp-black to the oil suspension. The values arrived at must therefore be taken only as minimum values.

The material to be analysed will be: (1) the daily eliminated amount of urine and of faeces; (2) parts of muscles containing the remaining deposits of the metal compounds injected; (3) the most important viscera; (4) in some cases the total remaining parts of the rabbit's body.

The results arrived at are given below, partly in numbered columns, partly—to simplify the survey—in a series of diagrams. The following descriptions will serve to explain these latter.

(1) The oblong rectangular columns show how much metal is contained in the amount of metal compound examined which is injected on the day

stated by the dates under the columns. A scale for the amount of metal will be found on the left side of each diagram. Where an analysis has failed it is marked with a question mark.

(2) The shaded area of each column represents the amount of metal, which was found, by analysis of the muscular tissue, to be unabsorbed.

(3) The lowest black transverse column gives the quantity found daily in the urine.

(4) The upper transverse column gives the quantity of metal contained in the faeces. Analyses which failed are shown by vertical lines. An empty space shows that no faeces were passed that particular day.

(5) The black oblongs at the top show the metal contained in the different viscera and blood. The tall black column or pillar found on the right hand of some of the diagrams gives the amount of metal contained in the remaining portions of the body.

Mercury.

The analytical method used comprised destruction of organic substances, precipitation with sulphuretted hydrogen, solution of the precipitate and electrolysis on thin gold electrodes, which were weighed on a Nernst microbalance.

The method possesses an accuracy of 0·03 mg.

A detailed account of the method is given by Lomholt and Christiansen [1917].

Four different Hg-compounds were investigated, each representing one of the four principal groups of mercurial compounds used in the treatment of syphilis.

Mercurium benzoicum. A 2 % water solution of mercurium benzoicum was used. 0·75 cc. of this was given each time. As the rabbit showed slight inflammation of the kidneys after injection No. 2, the injections were stopped for some days. The result of the analysis will be clear by the accompanying table (Diagram 1).

The preparation is absorbed quite regularly and rather quickly. The elimination curve of the urine accords well with this. A decided rise is not perceptible until towards the end, after several injections have been given on a number of consecutive days. The elimination in the faeces is much smaller than in the urine, and remains fairly constant during the period.

Acidum mercurisalicylicum. An injection of a 15 per cent. suspension in olive oil was given on alternate days. The injected places in the muscles were in some cases clearly defined, in others but very faintly. Consequently the amount of Hg found here was probably somewhat too small. But no doubt it is a preparation, which is absorbed very quickly. Moreover the elimination in urine and faeces soon becomes very considerable (Diagram 2).

Calomel. A 20 % suspension of calomel (*vapore paratum*) was used. The injected places appeared well defined against the sound muscle tissues, and

Rabbit No. 1. (Diagram 1.) Weight: 4200 g.
Compound injected: Mercurium benzoicum (2 % solution).

Elimination.

Date	Injected Hg mg.	Hg found 8. i. (unabsorbed) mg.	Hg found in the urine mg.	Hg found in the faeces mg.	Total quantity of eliminated Hg (mg.)
28. xii.	6·3	0·40			
29.			0·21	0·31	0·52
30.	6·3	2·11	0·43	0·14	0·57
31.			0·15	0·16	0·31
1. i.			0·05	0·11	0·16
2.			0·16	0·08	0·24
3.			0·32	0·14	0·46
4.	6·3	3·21	0·25	0·16	0·41
5.			0·23	0·23	0·46
6.	6·3	3·78	0·39	0·23	0·62
7.	6·3	5·20	0·34	0·05	0·39
8.	6·3	6·00	1·87	0·20	2·07
		20·70	4·40	1·81	6·21
			67·59 %	32·41 %	

Circulation of Hg.

	Weight of the organs (g.)	Quantity of Hg. found (mg.)	Contents per 100 g.
Heart	17	0·08	0·47
Lungs	18	0·06	0·33
Liver	118	0·57	0·49
Kidneys	20·5	0·45	2·20
Small intestine + faeces	121	0·47	0·39
Large „ „	115	0·36	0·32
Blood	110	0·13	0·12
Other part of the rabbit		4·80	
		6·92	

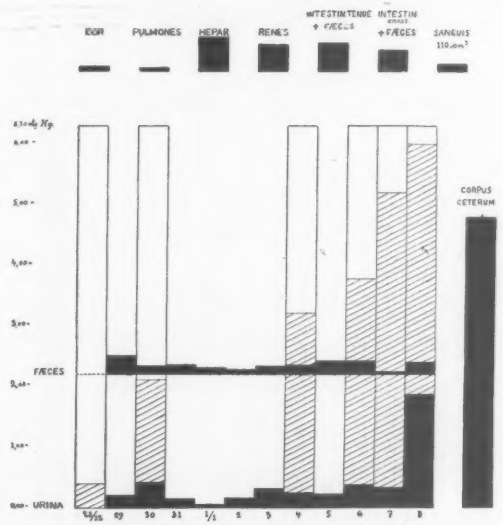

Diagram 1. Mercurium benzoicum.

Rabbit No. 2. (Diagram 2.) Weight: 2160 g.
Compound injected: Acidum mercurisalicylicum.

Elimination.

Date	Injected Hg mg.	Hg found 16. x. (unabsorbed) mg.	Hg found in the urine mg.	Hg found in the faeces mg.	Total quantity of eliminated Hg (mg.)
7. x.	4·4	0·10			
8.			0·63	0·05	0·68
9.	4·4	?	0·41	0·31	0·72
10.			1·63	0·38	2·01
11.	5·8	1·12	1·44	0·13	1·57
12.			0·52	0·48	1·00
13.	5·8	0·81	0·42	0·29	0·71
14.			0·63	0·41	1·04
15.	7·3	5·41	0·67	0·33	1·00
16.	5·8	4·10	0·72	0·43	1·15
		11·54	7·07	2·81	9·88
			71·55 %	28·45 %	

Circulation of Hg.

	Weight of the organs (g.)	Quantity of Hg found (mg.)	Contents per 100 g.
Lunga	22	0·05	0·23
Liver	75	0·87	1·16
Kidneys	17·5	0·88	5·03
Small intestine + faeces	60	0·33	0·55
Large „ „	90	0·48	0·53
Blood	55	0·11	0·20
Other part of the rabbit		6·40	
		9·12	

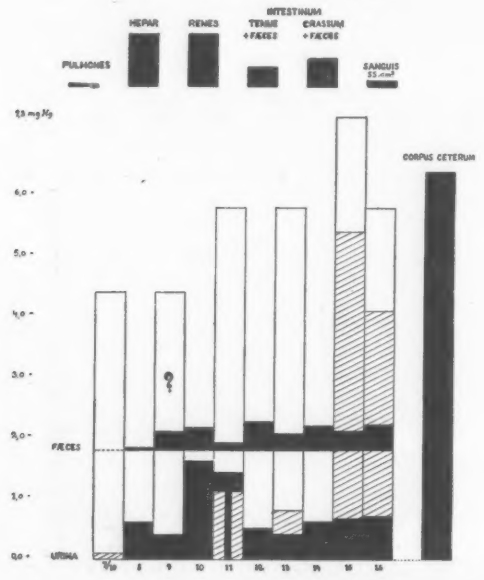

Diagram 2. Acidum mercurisalicylicum.

Rabbit No. 3. (Diagram 3.) Weight: 3900 g. Compound injected: Calomel.

Elimination.

Date	Injected Hg mg.	Hg found 20. i. (unabsorbed) mg.	Hg found in the urine mg.	Hg found in the faeces mg.	Total quantity of eliminated Hg (mg.)
11. i.	8·0	3·1			
12.			0·11	0·15	0·26
13.			0·18	0·13	0·31
14.	8·0	4·3	0·23	0·10	0·33
15.			0·32	0·16	0·48
16.	8·0	3·8	0·34	0·15	0·49
17.			0·42	0·10	0·52
18.	8·0	4·6	0·40	0·23	0·63
19.	8·0	4·6	—	0·16	0·16
20.	8·0	5·9	1·05	0·25	1·30
	26·3		3·05	1·43	4·48
			68·08 %	31·92 %	

Circulation of Hg.

	Weight of the organs (g.)	Quantity of Hg found (mg.)	Contents per 100 g.
Heart	13	0·21	1·61
Lungs	40	0·10	0·25
Liver	70	0·26	0·37
Kidneys	16	0·34	2·12
Small intestine + faeces	63	0·12	0·19
Large " "	140	0·27	0·20
Blood	75	0·10	0·13
Other part of the rabbit		6·40	
		7·80	

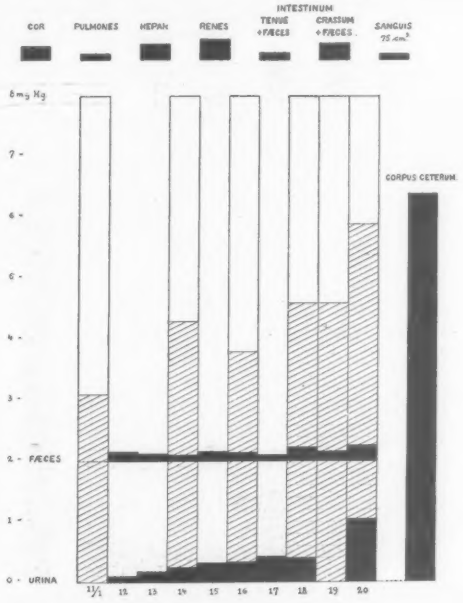

Diagram 3. Calomel.

were easy to remove. The analysis shows that the absorption takes place slowly, but very regularly. A corresponding regularity is also shown in the elimination in the urine. This rises regularly, while the elimination in faeces is considerably less, and rises but slightly (Diagram 3).

Oleum cinereum. A preparation of 50 % metallic mercury finely divided in oil; of this a quantity equivalent to 22 mg. Hg was given every third day. The first places injected were well defined, but not the later ones. The results show a very slow absorption.

Rabbit No. 4. (Diagram 4.) Weight: 4350 g.
Compound injected: Metallic mercury (50 % oil suspension).

Elimination.

Date	Injected Hg mg.	Hg found 6. ii. (unabsorbed) mg.	Hg found in the urine mg.	Hg found in the faeces mg.	Total quantity of eliminated Hg (mg.)
20. i.	22	13·8			
21.			0·05	0·05	0·10
22.			0·05	0·05	0·10
23.	22	14·8	0·11	0·08	0·19
24.			0·17	0·12	0·29
25.			0·28	0·14	0·42
26.	22	14·1	0·21	0·13	0·34
27.			0·36	0·23	0·59
28.			0·42	0·21	0·63
29.	22	16·3	0·30	0·17	0·47
30.			0·33	0·14	0·47
31.			0·38	0·13	0·51
1. ii.	22	18·8	0·51	—	0·51
2.			0·62	0·08	0·70
3.			0·58	0·08	0·66
4.	22	18·7	0·72	0·03	0·75
5.	22	19·7	0·67	0·03	0·70
6.			0·81	0·12	0·93
		116·2	6·57	1·79	8·36
			78·59 %	21·41 %	

Circulation of Hg.

	Weight of the organs (g.)	Quantity of Hg found (mg.)	Contents per 100 g.
Heart	18	0·16	0·89
Lungs	35	0·07	0·20
Liver	65	0·42	0·65
Kidneys	16	0·28	1·75
Small intestine + faeces	75	0·22	0·29
Large „ „	140	0·18	0·13
Blood	90	0·09	0·10
		1·42	

Experiments on patients [Lomholt, 1919, 1920] have shown that the absorption can be extremely irregular.

The elimination in the urine showed a slow, but regular rise corresponding to the absorption. In the faeces far less is eliminated and there was no regular rise (Diagram 4).

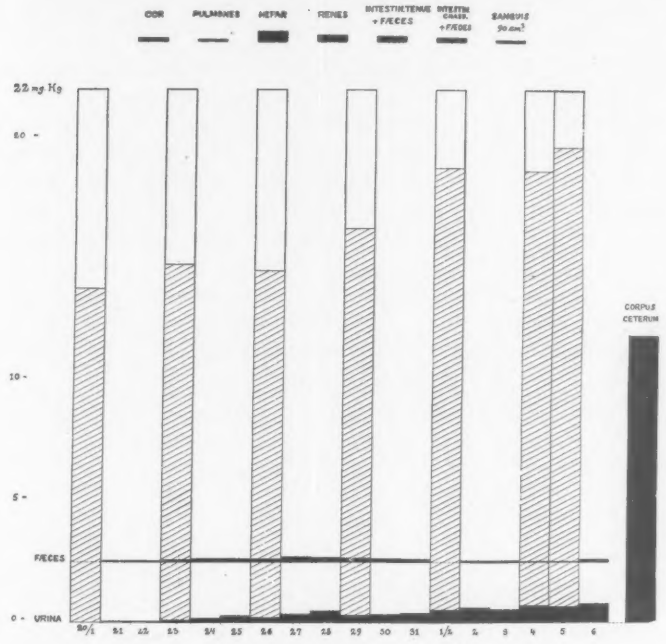

Diagram 4. Oleum cinereum (Hg).

The Distribution of Mercury in the Organism (Diagram 15).

There is an unmistakable conformity in all the experiments in the proportion between the amounts eliminated in the *urine* and those eliminated in the *faeces*. The urine constitutes in Exps. 1 and 2, 70 % of the collective amount; in 3, 68 %; and in 4, 79 %. This agrees with the fact that the *kidneys*, in percentage, are the organs by far the richest in metal. They contain about the same quantity as the liver although the latter is about six times as large. Thus the percentage in the kidneys is far greater than in the liver.

After the kidneys comes the *liver*. This must be regarded as the second most important organ for the elimination of mercury, rather than the great intestine, as is the opinion of many. If one considers the accompanying analyses of the small and large intestines, with their contents, it can be seen that in each of these considerable amounts are found. If the large intestine were the principal part for elimination of the mercury found in the faeces, one would expect to find small amounts of Hg only in the small intestine. But we always find considerable quantities, and these come assuredly from the liver. The *lungs* contain only a small amount of mercury. The same is

the case with the *heart*. Also in the *blood* there are only extremely small quantities found.

A considerable amount of metal was found in the remaining portion of the body of the rabbits. But one may hardly suppose that all this mercury was really absorbed from the injected places, and from thence carried to the tissue. Some portion has been removed most likely in a purely mechanical manner, a little way from the injected spot, and therefore has not been included in the excision.

The percentage distribution of the mercury in the organism is almost uniform in all the four investigated metal-compounds, but the actual amount of mercury found is proportionately far greater with the quickly absorbed preparations (mercurium benzoicum and acidum mercurisalicylicum) than with calomel, and especially with metallic mercury.

Bismuth.

An exact estimation of small quantities of mercury is very difficult. But it is still more difficult to estimate small amounts of bismuth. However, by the use of the radio-chemical method, first suggested by Hevesy and Paneth [1922], it becomes rather a simple matter, and also very exact. Small quantities of the radio-active bismuth isotope, radium E, are mixed with the quantity of inactive bismuth that is to be used in the experiment. When the radio-activity of the mixture is known, it is possible always to estimate the quantity of bismuth in an analysis, by simply determining the β-radiation of this, *i.e.* of the radium E contained in it. The proportionate mixture between active and inactive bismuth is in every case absolutely constant, and unchanged by anything whatever.

The organic substances are simply burnt away, the ash is spread out upon a shallow glass bowl, dried, and the β-radiation estimated [Christiansen, Hevesy and Lomholt, 1924].

The method permits of a good degree of exactitude when the radio-activity is not very small. Here the period of half decay—which for radium E is five days—plays a considerable part. Longer than after four periods, we have not undertaken any determination. The quantity of radium E used in each experiment was that produced by the disintegration of the emanation corresponding to a quantity of radium of 2–4 curies.

The two following compounds of bismuth were examined: bismuth quinine iodide (Viochine) and bismuth hydroxide.

The procedure was altogether parallel with that of the mercury experiments. Regard for the great quantities of ash hindered us from undertaking the estimation of bismuth in the remaining portions of the rabbit body. In the final experiment with $Bi(OH)_3$ (Diagram 11) we carried out a preliminary precipitation with H_2S after 0·1 g. of inactive bismuth had been added and in this way succeeded in estimating this amount also.

Rabbit No. 5. (Diagram 5.) Weight: 1900 g. Compound injected: Viochine.

Elimination.

Date	Injected Bi mg.	Bi found 11. ii. (unabsorbed) mg.	Bi found in the urine mg.	Bi found in the faeces mg.	Total quantity of eliminated Bi (mg.)
1. ii.	2·65	1·20			
2.	2·65	1·34	0·07	0·01	0·08
3.	2·65	1·88	0·31	0·01	0·32
4.	2·65	?	0·31	—	0·31
5.	2·65	2·07	0·25	0·16	0·41
6.	2·65	2·55	?	0·16	0·16
7.	2·65	1·97	0·35	0·31	0·66
8.			0·32	0·21	0·53
9.			?	0·18	0·18
10.			0·39	0·11	0·50
11.			0·17	—	0·17
		11·01	2·17	1·15	3·32
			65·36 %	34·64 %	

Circulation of Bi.

	Weight of the organs (g.)	Quantity of Bi found (mg.)	Contents per 100 g.
Heart	8	0·01	0·12
Lungs	9	0·01	0·11
Liver	78	0·08	0·10
Kidneys	16·5	0·35	2·00
Small intestine + faeces	?	?	?
Large „ „	?	?	?
Blood	50	0·02	0·04
		0·47	

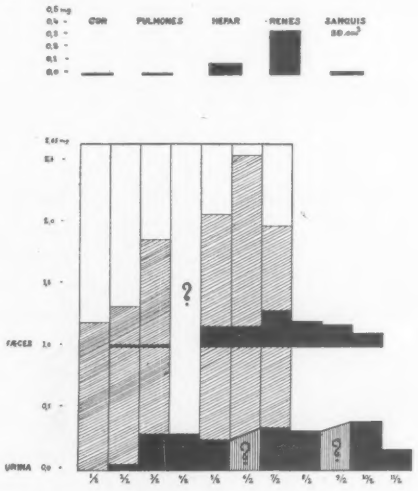

Diagram 5. Viochine.

Rabbit No. 6. (Diagram 6.) Weight: 1500 g. Compound injected: Viochine.

Elimination.

Date	Injected Bi mg.	Bi found 24. ii. (unabsorbed) mg.	Bi found in the urine mg.	Bi found in the faeces mg.	Total quantity of eliminated Bi (mg.)
15. ii.	2·5	1·50			
16.	2·5	0·76	0·08	0·05	0·13
17.	2·5	1·52	0·03	0·05	0·08
18.	2·5	?	0·04	0·04	0·08
19.	2·5	0·79	0·07	0·06	0·13
20.	2·5	1·52	0·04	0·08	0·12
21.	2·5	1·50	—	—	—
22.	2·5	1·55	0·20	0·07	0·27
23.	2·5	0·74	0·21	0·07	0·28
24.			0·62	0·05	0·67
		9·88	1·29	0·47	1·76
			73·30 %	26·70 %	

Circulation of Bi.

	Weight of the organs (g.)	Quantity of Bi found (mg.)	Contents per 100 g.
Heart	4	0·01	0·25
Lungs	7·5	0·02	0·27
Liver	50	0·05	0·10
Kidneys	10	0·14	1·40
Small intestine—faeces	?	0·11	?
Large „ „	?	?	?
Blood	35	0·01	0·03
		0·34	

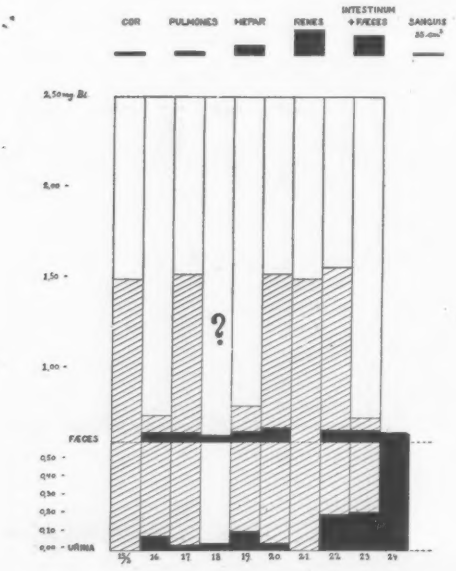

Diagram 6 Viochine.

Rabbit No. 7. (Diagram 7.) Weight: 1770 g. Compound injected: Viochine.

Elimination.

Date	Injected Bi mg.	Bi found 2. iv. (unabsorbed) mg.	Bi found in the urine mg.	Bi found in the faeces mg.	Total quantity of eliminated Bi (mg.)
20. iii.	1·55	0·51			
21.	1·55	0·75	0·01	0·03	0·04
22.	1·55	0·72	0·03	0·01	0·04
23.	1·55	1·01	0·06	0·03	0·09
24.	1·55	0·39	0·08	0·03	0·11
25.	1·55	0·42	0·08	0·08	0·16
26.	1·55	0·86	0·08	0·05	0·13
27.	1·55	0·75	0·17	0·06	0·23
28.	1·55	1·15	0·20	0·05	0·25
29.	1·55	1·13	0·22	0·08	0·30
30.			0·14	0·05	0·19
31.			0·14	0·04	0·18
1. iv.			0·13	0·04	0·17
2.			0·13	0·04	0·17
		7·69	1·47	0·59	2·06
			71·36 %	28·64 %	

Circulation of Bi.

	Weight of the organs (g.)	Quantity of Bi found (mg.)	Contents per 100 g.
Heart	5	0·01	0·20
Lungs	8	0·01	0·13
Liver	72	0·05	0·07
Kidneys	11	0·10	0·90
Small intestine + faeces	65	0·08	0·12
Large „ „	110	0·09	0·08
Blood	45	0·01	0·02
		0·35	

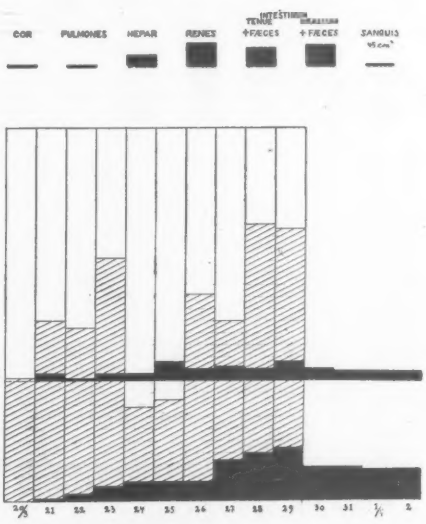

Diagram 7. Viochine.

Rabbit No. 8. (Diagram 8.) Weight: 2680 g.
Compound injected: Bismuth hydroxide.

Elimination.

Date	Injected Bi mg.	Bi found 27. iv. (unabsorbed) mg.	Bi found in the urine mg.	Bi found in the faeces mg.	Total quantity of eliminated Bi (mg.)
13. iv.	3·80	1·22			
14.	3·80	0·77	0·14	0·00	0·14
15.	3·80	0·56	0·28		0·28
16.	3·80	0·38	0·20	0·14	0·34
17.	3·80	1·87	0·26	0·23	0·49
18	3·80	0·47	0·30	0·09	0·39
19.	3·80	0·84	0·49	0·23	0·72
20.	3·80	0·48	0·35	0·21	0·56
21.	3·80	1·59	0·35	0·19	0·54
22.			0·28	0·14	0·42
23.			0·42		0·42
24.			0·33	0·40	0·73
25.			0·23	0·16	0·39
26.			0·44	0·14	0·58
27.			0·30	0·42	0·72
		8·18	4·37	2·35	6·72
			65·03 %	34·97 %	

Circulation of Bi.

	Weight of the organs (g.)	Quantity of Bi found (mg.)	Contents per 100 g.
Heart	9	0·12	1·33
Lungs	17	0·07	0·41
Liver	102	?	?
Kidneys	19	0·56	2·94
Small intestine + faeces	?	?	?
Large » »	?	?	?
Blood	50	0·16	0·32
		0·91	

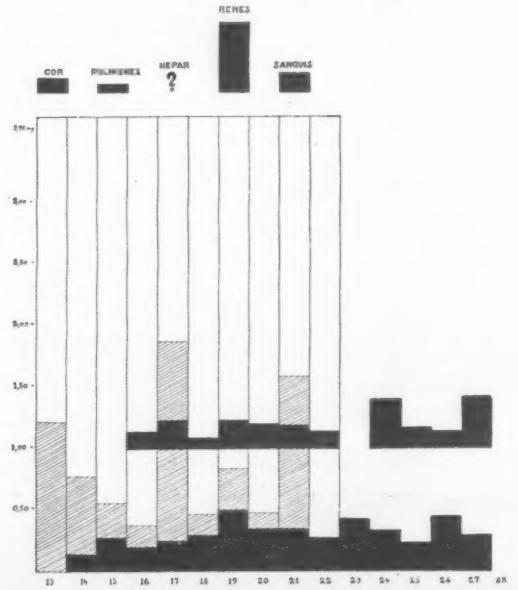

Diagram 8. I. $(BiOH)_3$.

Rabbit No. 9. (Diagram 9.) Weight: 2460 g.
Compound injected: Bismuth hydroxide.

Elimination.

Date	Injected Bi mg.	Bi found 25. v. (unabsorbed) mg.	Bi found in the urine mg.	Bi found in the faeces mg.	Total quantity of eliminated Bi (mg.)
14. v.	16	1·5			
15.	16	3·0	0·06	0·00	0·06
16.	16	4·0	0·30	0·04	0·34
17.	16	?	0·36	0·06	0·42
18.	16	?	0·09	0·16	0·25
19.	16	14·0	0·51	0·09	0·60
20.	16	?	0·54	0·15	0·69
21.	16	13·0	0·53	0·22	0·75
22.			0·54	0·34	0·88
23.			0·87		0·87
24.			0·86	0·17	1·03
25.			0·40	0·08	0·48
	35·5		5·06	1·31	6·37
			79·43 %	20·57 %	

Circulation of Bi.

	Weight of the organs (g.)	Quantity of Bi found (mg.)	Contents per 100 g.
Heart	7	0·01	0·14
Lungs	15	0·04	0·27
Liver	127	0·12	0·09
Kidneys	17	0·37	2·17
Small intestine + faeces	?	?	?
Large „ „	?	?	?
Blood	70	0·01	0·014
		0·55	

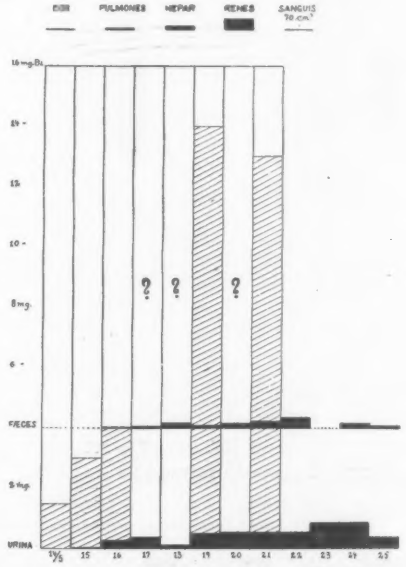

Diagram 9. II. $Bi(OH)_3$.

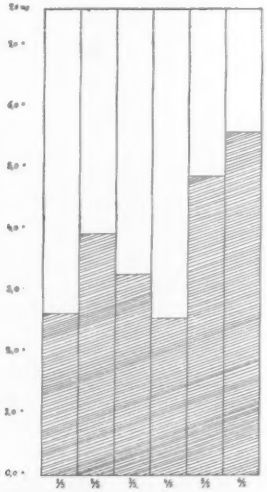

Diagram 10. III. $Bi(OH)_3$.

The two preparations investigated appeared to offer only slight variations. On one particular point they offered somewhat greater difficulties than the mercury experiments.

Bismuth causes less reaction in the muscular tissue than mercury. The injected areas are therefore less well defined and especially with the deposits of $Bi(OH)_3$. In the two first experiments we found surprisingly low, irregular values for these latter deposits, which could only be explained by the excision having been incomplete. We then tried mixing a little lampblack with it, and found more satisfactory values (III and IV, Diagrams 10 and 11), as we could more easily follow the trail of the black specks.

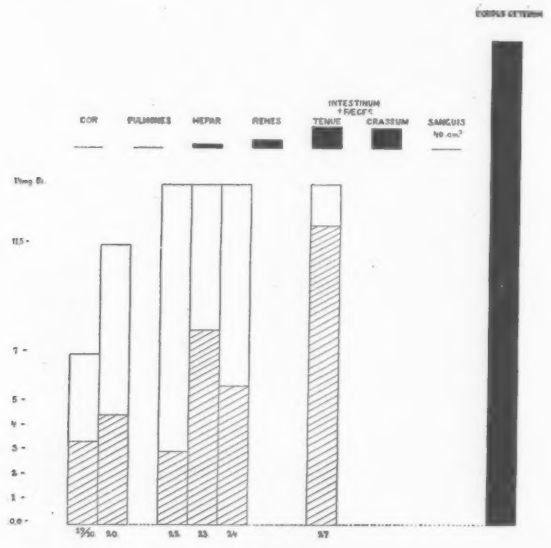

Diagram 11. IV. $Bi(OH)_3$.

Three experiments were made with viochine (Diagrams 5, 6, 7) there being a daily injection of a small quantity of this compound. All these three experiments resembled each other greatly. The absorption takes place fairly slowly, but sometimes almost capriciously (Diagram 7).

Of the four hydroxide experiments (Diagrams 8, 9, 10, 11) the first two show, as regards the injected deposit, very great irregularities (see above). Exps. III and IV are in this respect remarkably better, but even they are not fully dependable. The very large amount of bismuth found in the remainder of the body in Exp. IV points to this. A considerable part of this assuredly comes from the unabsorbed quantities in the surroundings of the injected parts.

The elimination in urine and faeces shows conditions very much like those in the mercury experiments. The chief part is eliminated in the urine (about $\frac{2}{3}$) and the elimination rises during the experiments, though often with considerable irregularity. As regards the faeces the values were much smaller and especially the rise was less pronounced; the irregularities somewhat more than in the urine.

The elimination was studied in five cases. In the first four the proportion between injected and eliminated Bi was much the same. In the fifth (Diagram 9) the elimination was proportionally small. Probably this is due to the fact that the total quantity injected here was about six times as large as in the other four experiments.

The distribution of bismuth in the organism (Diagram 15).

The distribution proved to be very much alike in all the seven experiments.

The greatest amount of bismuth is always found in the *kidneys*; not only relatively, but also positively. As a rule about twice as much is found in the kidneys as in the liver, though this latter is six or seven times as large.

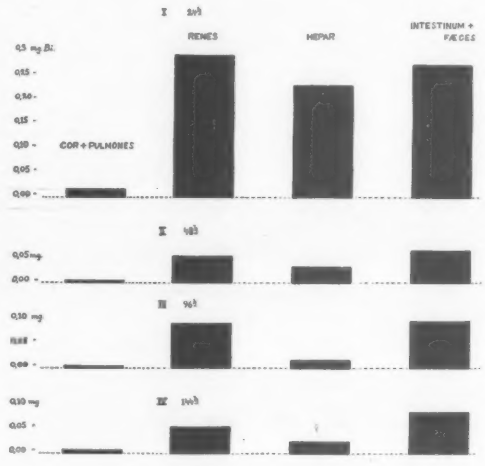

Diagram 12. Bi.

This is in perfect harmony with the fact, that far the greater part of the bismuth leaves the body through the kidneys, while the intestines come in the second rank. The chief part of this bismuth comes without doubt from the *liver*, as is the case with mercury. Unfortunately I have only two experiments with the great and small *intestines*; but they both agree. The amount in *heart* and *lungs* is minimum, and the same is the case with the *blood*.

I found an excellent corroboration of this in a series of experiments on guinea-pigs (Diagram 12). I injected 10 mg. Bi in a very weak solution of

nitrate into the muscular tissue of four guinea-pigs of a like size, and at the same time. Twenty-four hours later one was killed; after two days another; after four days the third; and after six days the fourth. The results are given on Diagram 12 and hardly require any comment.

It will be seen easily that the bismuth compounds and the mercury compounds (and especially calomel) resemble each other very much as regards circulation and elimination.

This agrees perfectly with their well-known resemblance to each other in other points; they cause the same symptoms of intoxication (nephritis, stomatitis and colitis) and possess the same peculiar effect on syphilis.

Lead (Diagrams 13 and 14).

The analytical method used was quite parallel with that in the bismuth experiments, only that instead of radium E an isotope of lead (radium D) was employed [Hevesy, 1923].

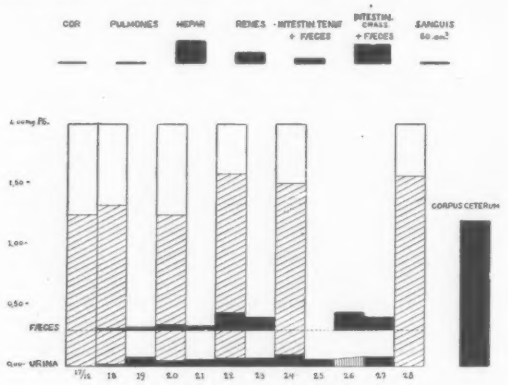

Diagram 13. I. $Pb(OH)_2$.

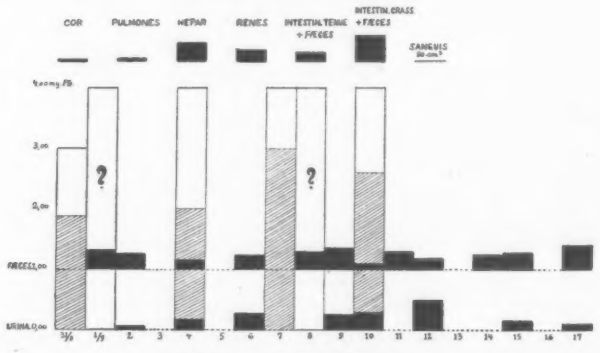

Diagram 14. II. $Pb(OH)_2$.

Only one lead compound was investigated and only two experiments on rabbits took place, which however gave results agreeing well with each other. Exp. 1 was in particular very successful. Exp. 2 was hindered by irregularities, as the rabbit expelled both urine and faeces in a quite unusually irregular way.

To the emulsion used, lamp-black was added. This is probably the reason that the investigations of the injected deposits gave rather good results. This goes to prove slow, but quite regular absorption.

The conditions of elimination are best seen in Exp. 1. In the urine it is very gentle and regular. That in the faeces is considerably less regular. But what specially catches our attention is, that the amount eliminated by this way is greater than that by the kidneys (52·8 % against 47·2 %). In Exp. 2 this is still more noticeable.

Rabbit No. 12. (Diagram 13.) Weight: 2300 g.
Compound injected: Lead hydroxide.

Elimination.

Date	Injected Pb mg.	Pb found 28. xii. (unabsorbed) mg.	Pb found in the urine mg.	Pb found in the faeces mg.	Total quantity of eliminated Pb (mg.)
17. xii.	2·0	1·25			
18.	2·0	1·33	0·02	0·02	0·04
19.			0·08	0·03	0·11
20.	2·0	1·25	0·05	0·05	0·10
21.			0·06	0·04	0·10
22.	2·0	1·59	0·07	0·15	0·22
23.			0·07	0·11	0·18
24.	2·0	1·51	0·10		0·10
25.			0·06		0·06
26.			?	0·15	0·15
27.			0·08	0·11	0·19
28.	2·0	1·57	?		
	8·50		0·59	0·66	1·25
			47·2 %	52·8 %	

Circulation of Pb.

	Weight of the organs (g.)	Quantity of Pb found (mg.)	Contents per 100 g.
Heart	6	0·01	0·17
Lungs	12	0·01	0·08
Liver	120	0·19	0·16
Kidneys	15	0·09	0·60
Small intestine + faeces	120	0·04	0·03
Large „ „	150	0·16	0·11
Blood	50	0·01	0·02
Other part of the rabbit	1700	1·20	
		1·71	

Rabbit No. 13. (Diagram 14.) Weight: 2730 g.
Compound injected: Lead hydroxide.

Elimination.

Date	Injected Pb mg.	Pb found 17. ix. (unabsorbed) mg.	Pb found in the urine mg.	Pb found in the faeces mg.	Total quantity of eliminated Pb (mg.)
31. viii.	3·0	1·87			
1. ix.	4·0	?		0·33	0·33
2.			0·06	0·26	0·32
3.					
4.	4·0	2·00	0·17	0·18	0·35
5.					
6.			0·28	0·24	0·52
7.	4·0	3·00			
8.	4·0	?		0·30	0·30
9.			0·25	0·37	0·62
10.	4·0	2·80	0·31	0·11	0·42
11.				0·29	0·29
12.			0·44	0·18	0·62
13.					
14.				0·22	0·22
15.			0·18	0·28	0·46
16.					
17.			0·10	0·41	0·51
	9·67		1·79	3·17	4·96
			36 %	64 %	

Circulation of Pb.

	Weight of the organs (g.)	Quantity of Pb found (mg.)	Contents per 100 g.
Heart	7	0·04	0·57
Lungs	10	0·05	0·50
Liver	55	0·30	0·55
Kidneys	12	0·20	1·67
Small intestine + faeces	?	0·15	?
Large „ „	?	0·44	?
Blood	50	0·01	0·02
		1·19	

The Distribution of Lead in the Organism. (Diagram 15.)

In agreement with the above-mentioned facts as to the conditions of elimination, there is found here a considerably greater amount of metal in the *liver* than in the *kidneys*, about twice as much. In *heart*, *lungs* and *blood* only quite a small amount is found. The great intestine contains in both cases more than double as much as the small intestine. Nevertheless it will be natural also here to regard the liver as that part from which the chief amount of the lead found in the evacuation comes. Faeces often pass through the great intestine very slowly.

Summary.

The three metals investigated showed much the same conditions in the organism. Still, there seems to remain a slight difference. With bismuth, the kidneys (and the urine) play by far the most prominent rôle, with lead,

the liver (and the faeces) seems to be just as important. Mercury comes between the two, but somewhat nearer to bismuth than to lead.

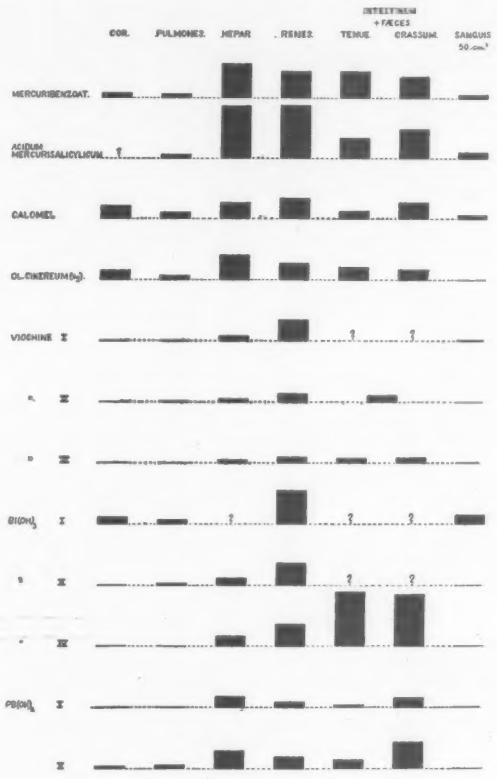

Diagram 15. Viscera.

I wish to express my most cordial thanks to Dr Christiansen and Prof. Hevesy for their excellent and extensive help in the above investigation.

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XCIII. THE SYNTHESIS OF STARCH FROM SUGAR BY BACTERIA.

By EGERTON CHARLES GREY.

From the Chemical Department of the Government Medical School, Cairo.

(*Received April 21st, 1924.*)

Granules staining blue with iodine have been noticed in higher fungi, bacteria and in protozoa, but the nature of the substance and its significance is not definitely known. The substance formed by *B. coli* is shown in the present communication to be no other than starch, and the writer is of the opinion that the proof of the existence of starch in bacteria is of the highest significance.

Starch is readily detected in the early stages of the fermentation of glucose in presence of chalk by bacteria. It is present in the solid bacterial debris, which settles together with the calcium oxalate precipitate when oxalic acid is added in the process of removing calcium after the fermentation, and it is present throughout the medium in the bacterial particles. After prolonged boiling with oxalic acid, in the process of distilling the volatile acids, it appears abundantly in the solution as soluble starch. It has been found present most abundantly from about the sixteenth to the fortieth hour of the fermentation. At some periods there appears to be an almost complete transformation of glucose into non-reducing carbohydrate, a great proportion of which has been shown to be starch. The solutions after removal of the various products of decomposition yield precipitates upon the addition of alcohol. The soluble starch gives the characteristic blue coloration with iodine, and shows a high dextrorotation, the extent of which depends upon the treatment by which the soluble starch has been formed from the original starch of the bacteria. Gentle treatment in the cold yields a soluble starch of very high rotation. Prolonged boiling with oxalic acid yields a soluble starch or amylodextrin showing a dextrorotation, $[a]_D = 200°$. The soluble starch of highest rotation showed no reduction with copper solutions. Samples of lower rotation showed in some cases reduction. It yields glucose on hydrolysis. I have not so far been able to find any glycogen.

The discovery of the production of vegetable starch by bacteria establishes still further my thesis that the fermentation processes of many different organisms are *au fond* very similar, a thesis which I desire to maintain because I believe that many physiological problems will be more readily solved by the study of the so-called lower organisms than the higher. I have for some time tried to emphasise the importance of the synthetic side of bacterial fermentation, and I hope that the demonstration of the production of vegetable starch will convince more workers as to the importance of this line of enquiry.

Since it is well known [see Harden, 1923] that the non-reducing carbohydrate of yeast is glycogen, it is of special interest that with *B. coli*, I find only starch, and I venture to suggest the possibility that some yeasts may synthesise starch also. This enquiry is being taken up. That there was an abundant formation of a non-reducing carbohydrate in the early stages of a bacterial fermentation was reported by myself in a paper from the Pasteur Institute [1918]. I did not know then that the substance was starch. It is probable however that there are several polysaccharides formed but they are not demonstrable by iodine, and cannot be so definitely characterised.

Experimental.

The essence of the experiment consists in removing samples during the early stages of a fermentation. The fermentation is brought about by mixing an emulsion of bacteria (which contains a certain amount of soluble nitrogenous matter), with a solution of glucose containing about 0·6 % of salts. Chalk is added to neutralise the acids which are produced by the fermentation. The fermentation is carried out in absence of air, but the bacteria employed have been grown previously aerobically on agar.

Arrangements for removing the samples. The technique previously described has been still further simplified. The fermentation is now carried out in a large evacuated flask, floating up-side-down in a large water tank kept at a constant temperature of 37°; the flask is enveloped in a net and secured by string to the sides of the tank. There is thus no strain and the flask however large has virtually no weight. The neck of the flask which is submerged, is provided with a rubber stopper through which two tubes pass, one into the liquid in the flask and the other into the gas space above. The rate of appearance of the fermentation gases in the evacuated flask may be measured by a mercury manometer. Since there are no joints above water and the flask is under reduced pressure there can be no question as to any gases being lost. In fact, it may be mentioned, that the idea in mind in adopting this arrangement was to investigate certain problems in connection with the gas production. These points will however not be discussed here.

In the experiment about to be described a ten litre flask was used and the volume of the fermenting liquid was three litres. Samples of about 500 cc. were removed at about six hourly intervals.

The samples are removed by connecting to the fermentation flask an evacuated bottle of the requisite size. Simultaneously with the removal of the liquid sample, a gas sample is removed, by the other tube which is connected to a mercury burette.

Experiment carried out February 20th–22nd, 1924. Organism of coliform type, in pure culture, with the following characteristics. Slightly motile, Gram negative, fermenting lactose, glucose, mannitol, with the production of acid and gas. Not fermenting dulcitol.

Composition of the fermenting fluid at the beginning of the experiment.

Glucose	60·4 g.
Bacteria	5·4 g.
Nitrogenous matter (mixed amino acids)	5·1 g.
Magnesium sulphate	3·0 g.
Potassium „	15·0 g.
Tap water	3 litres
Chalk added	25 g.

Analysis of the carbohydrate present at various periods.

	Time in hours from the start	Glucose before hydrolysis g.	Glucose after hydrolysis g.	Synthetic carbohydrate present g.
1	0	60·4	60·4	—
2	9	52·5	52·5	nil
3	16	31·7	32·1	0·4
4	22	15·2	16·3	1·1
5	33	3·2	5·8	2·6
6	39	nil	1·8	1·8

The synthetic carbohydrate is rapidly hydrolysed by the bacterial enzymes, and the hydrolysis in many cases is found to occur in the presence of antiseptics; this point will be referred to elsewhere. Since however there is a continual decomposition of the complex carbohydrate it is clear that the amount present at any time is merely the excess of what is formed over what is decomposed so that the amount of this material actually built up by the bacteria is probably very much greater than appears from the table. Allowing for the portions removed in sampling we see that 3·2 g. of synthetic carbohydrate was present in the fermentation fluid, and it was found that 35·5 g. of glucose had been fermented. This is a yield of 9 %. From the portion remaining after many analyses the starch was precipitated by the addition of alcohol and a weight of 0·60 g. was obtained, corresponding to 3·0 g. of starch in the total fermentation fluid. Starch was also present in the precipitates of calcium oxalate which are formed in the process of analysis, and mixed with the kieselguhr which is used for filtration, so that the amount obtained represents only a portion of the total.

The production of the synthetic carbohydrate measured by the optical rotation.

	Time (in hours)	Glucose % measured by the reduction method	Calculated rotation	Observed rotation (2 cm. tube)
1	9	1·68	1·78	1·82
2	16	1·33	1·49	1·72
3	22	0·91	0·96	1·54
4	33	0·46	0·48	0·93
5	39	0·32	0·33	0·69

The synthetic carbohydrate has clearly a high rotatory power. An actual measurement upon a sample of soluble starch isolated from the fermentation fluid gave the following result.

Observed in a length of 1 dm, a 0·712 % solution gave a rotation of 1·4°, $[\alpha]_D = 197°$. This material was quite soluble in cold water, and had all the properties of soluble starch, it had however been obtained by prolonged boiling of the fermentation fluid which contained bacteria in suspension. I cannot say definitely whether any truly soluble starch exists preformed in the bacteria, there is none free in the fermentation fluid prior to boiling, but as it is so readily fermented it would doubtless be difficult to detect even if it escaped from the bacteria. The greatest part of the bacterial starch however is undoubtedly present in the substance of the bacteria and is liberated from them on prolonged boiling of the solution in which they are suspended.

The major part of the starch obtained in this experiment had already been degraded to a form of soluble starch or amylodextrin before I was aware of its nature. This was the result of the prolonged boiling with oxalic acid solution which forms part of the routine analysis for the fermentation products. Some of the samples obtained possessed a definite reducing power, whilst others were quite devoid of any reducing power. The rotation of the latter was higher than that of those which, while giving a blue colour with iodine, also showed some reduction with copper solutions. A solution which showed no reduction was found to have an $[\alpha]_D$ of the order of 500°. This solution was obtained by adding hydrochloric acid to a bacterial sediment which contained chalk, and extracting the emulsion with ether, in a continuous apparatus; this treatment liberated the starch into the solution in a soluble form. A 0·01 % solution in a 20 cm. tube gave a rotation of 0·10°. The solution yielded a precipitate with alcohol, and after hydrolysis yielded phenylglucosazone of typical form.

Conclusions.

Bacteria synthesise starch abundantly from glucose. The synthetic side of bacterial metabolism is just as important as the decomposition which is such a characteristic feature. Probably a very large proportion of the various substances which are decomposed by bacterial fermentation are first synthesised into more complex materials within the bacterial protoplasm, and only subsequently undergo a decomposition into the end products which are commonly observed.

To study the formation of starch and similar substances by bacteria the fermentation must be interrupted at a very early stage. In the experiments described the synthetic period was passed in about 40 hours.

I wish to return my best thanks to my colleague W. M. Colles who has helped me in making many of the observations.

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XCIV. THE INFLUENCE OF DIET AND SUNLIGHT UPON THE GROWTH-PROMOTING AND ANTI-RACHITIC PROPERTIES OF THE MILK AFFORDED BY A COW.

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I. Introduction.

The effect of diet on the growth-promoting properties of cow's milk has been studied by several investigators. Dutcher and Kennedy [1921, 1922] investigated the influence of the diet of the cow on the quality of the milk as regards its content of vitamins A and B. They found that the vitamin content of the milk depended on the feeding of the animal, that both vitamins A and B could be supplied to a stall-fed cow by a proper combination of grain and leafy foods, and also that a vitamin-rich milk can only be obtained by feeding a cow on pasture, if the pasturage is fresh and green.

Drummond, Coward and Watson [1921] have shown that the growth-promoting value of butter varies with the season, and this is attributed by them to the difference in diet of cows during summer and winter. They have recently communicated the results of further experiments, in which they reduced the vitamin A content of milk by feeding the cow on a diet deficient

in this vitamin, and conversely increased it by the addition of cod-liver oil to the dietary. On the other hand, Hume [1923] was unsuccessful in improving the growth-promoting value of the milk of a stall-fed cow, although it was fed upon abundant green food for a period of three months. Following on her discovery that animals whose growth had become arrested upon a diet deficient in vitamin A, recommenced to grow at a normal rate, if irradiated with ultraviolet light for a few minutes daily, she explored the possibility that the milk of a cow in sunlight might show an enhanced value over that of an animal on the same diet in a dark stall. A cow was kept out of doors for 12 hours daily during the height of summer, but no significant improvement in the growth-promoting value of the milk was detected after two months. The diet was deficient in vitamin A and consisted of cereals, straw and roots.

In a recent paper [1924] Reyher gives clinical evidence that the milk from cows on fresh green fodder has a higher growth-promoting power than that from cows on dry fodder. He fed infants with milk from cows on green and dry fodder alternately, and found that their weight was arrested with surprising constancy when they received milk from cows on dry fodder.

I am unaware of any observations on the influence of diet upon the anti-rachitic properties of cow's milk, although, from analogy, it has been assumed that these would also be modified by the food supplied. This view is supported by the fact that it is impossible to produce rickets in litters of young rats for a considerable time after weaning, if the mother is well supplied with cod-liver oil during pregnancy and lactation, and conversely rickets is more easily induced, and in a severer form, in young rats when the mother has been subject to diet deficiencies during pregnancy and lactation [Korenchevsky, 1921, 1922, 1923].

The effect on the anti-rachitic value of the milk of exposing the cow to more or less sunlight has not, I believe, been investigated. After the recent discoveries of the influence of light in the prevention and cure of rickets in children and in animals, it would not be surprising should the anti-rachitic value of milk respond to variations in the radiant energy impinging upon the cow. The average anti-rachitic properties of cow's milk cannot, however, be very great, for many children reared upon it suffer from rickets. Indeed, whilst nearly every observer admits that cod-liver oil has a specific effect in the prevention and cure of rickets, there is divergence of opinion as to whether cow's milk possesses any anti-rachitic properties. Mellanby [1919, 1921] working with dogs, and Korenchevsky [1921, 1922] working with rats, however, agree that the withdrawal of milk fat from certain dietaries and its substitution by a vegetable oil determines the occurrence of rickets in these animals. Paton and Watson [1921], however, do not agree that milk fat can prevent rickets in puppies.

The experience of Hess and Unger [1920] is that children develop rickets in spite of the consumption of large amounts of cow's milk. The possibility

of the milk being of unequal value at different times of the year was not lost sight of by these workers, and in a later paper [1921] on the interpretation of the seasonal variation of rickets, they record observations on two groups of children, amongst those under their care in an institution, which were fed upon dried milks prepared from stall-fed and pasture-fed cows, respectively. Two or three out of each group developed mild rickets. Hess and Unger are evidently among those who consider milk to be devoid of anti-rachitic properties, for they formulate their conclusions thus: "the milk of cows supplied with green fodder does not acquire anti-rachitic properties." Findlay [1922] supports the view of Hess and Unger, because in his clinic many children on a diet poor in milk fat present no evidence of rickets, and some of the most severe examples of this disease occur in children fed on a diet rich in fat. The same conclusion was reached by Galbraith [1923] as a result of a large series of clinical observations.

During the clinical observations of Chick, Dalyell, Mackay and others [1923] on rickets in Vienna, children admitted with active rickets during winter and spring, were fed upon milk from stall-fed cows receiving different diets, including a diet of fresh green fodder. No healing was demonstrated in the bone lesions, in absence of other specific treatment.

The pathology of rickets in animals and in children is so essentially similar, that the discordance between clinical and experimental results demands explanation.

There are other factors which are now known to influence the development of rickets besides the presence or absence of a fat-soluble, anti-rachitic factor in diet. These are notably the degree of exposure to light and the absorption of Ca and P. It is evident that milk, unlike cod-liver oil, does not contain any superabundance of this anti-rachitic principle, and may, therefore, not have been the controlling influence in particular cases. This interpretation of clinical observations is consistent with experience in the laboratory. It is emphasised by it. Young rats receiving a ration of milk may become rachitic or not, according to the amounts of phosphorus in their diet, and the amount of direct sunlight to which they are exposed.

II. General method of experiment.

The following experiment was started in July, 1922, and continued until December, 1923. A fawn-coloured Jersey cow, which had previously been for three months on pasture, and was, at the beginning of the experiment, three months pregnant with her seventh calf, was kept during this time under carefully controlled conditions at the Serum Department of the Lister Institute in Herts. I am much indebted to Dr MacConkey, for kindly undertaking to arrange for the feeding and management of the cow, and for the collection and pasteurisation of the milk samples.

The observations may be divided into five periods.

Period I (July 5th–November 8th, 1922). *The cow was kept on a diet deficient in fat-soluble vitamins, but was out of doors in the sunshine.* She was kept out all day in a ploughed enclosure, and received daily: white maize meal, $3\frac{3}{4}$ lbs., gluten meal, 3 lbs.; oats and barley (crushed), 3 lbs.; mangolds, 50 lbs.; barley straw *ad lib.*

Period II (November 9th, 1922–May 24th, 1923). *Dry fodder in the dark.* The same diet was continued as in period I, but the cow was placed in a well-ventilated, but completely dark stall. The door of the stall was divided into an upper and lower half; the upper half was replaced by a louvre for purposes of ventilation, and remained fixed. The lower half was opened a short distance each day for purposes of cleaning and milking; for the remainder of the time it was tightly closed. No direct light entered the stall, and the conditions of darkness, therefore, were as complete as was compatible with the care and well-being of the animal.

Period III (May 25th–July 31st, 1923). *Green fodder in the dark.* During this time the conditions of darkness described in period II were continued, but the diet of the cow was changed to one of freshly cut meadow grass and clover, given in equal parts, of which she ate 120–130 lbs. per day.

Period IV (August 1st–October 22nd, 1923). *Pasture feeding.* She was turned out into the fields all day, and kept at night in a light shed, built in the pasture. The weather, especially during August, was bright and sunny, and the pasture fresh and green.

Period V (October 23rd–December 19th, 1923). The conditions described in period I were repeated.

The milk was tested at monthly intervals on rats. Equal samples of the whole morning's milking were taken, with sterile precautions, over a period of seven days. These were pooled, and CO_2 was bubbled through for five minutes, followed by pasteurisation for 20 minutes at 70° C. in an atmosphere of CO_2, to prevent oxidation of the fat-soluble vitamins. The milk was kept thereafter in the refrigerating chamber at about 0°. By this method it was found that the samples remained fresh for 8–10 weeks or even longer.

III. Technique employed for the evaluation of the milk.

1. *Determination of the growth-promoting value.*

Rats which had ceased to grow, from deprivation of vitamin A, were used for these tests. The rats were bred in the Institute from animals fed on a diet of fresh cow's milk, white bread, oats and bran, carrots or cabbage, with, occasionally, cooked meat. The same breeding diet was used throughout the experiment and the litters showed a fairly uniform response to the vitamin A deficiency in their diet.

The young rats were kept in a well-lighted and well-ventilated room at a temperature of 60–65° F. The windows faced N.N.E., and were kept shut;

the animals were not exposed to direct sunlight. For a preliminary period of 3–4 weeks the rats received a modification of the basal diet recommended by Zilva and Miura [1921] which consisted of the following ingredients: egg white (cooked), 20 %; starch, 50 %; salt mixture, 5 %; marmite, 5 %; lemon juice, 5 %; cotton-seed oil, 15 %; water 90 cc.

The salt mixture used was as follows:

Sodium chloride (NaCl)	51·9
Magnesium sulphate ($MgSO_4$, $7H_2O$) ...	164·0
Sodium acid phosphate ($NaH_2PO_4 + 2H_2O$)	104·1
Potassium phosphate (K_2HPO_4)	286·2
Calcium phosphate [$CaH_4(PO_4)_2 + H_2O$] ...	162·0
Calcium lactate [$(C_3H_5O_3)_2Ca + 5H_2O$] ...	390·0
Ferric citrate...	35·4

The basal diet contained 0·28 % Ca and 0·6 % P in the fresh diet. This has been shown by Korenchevsky [1922] to be sufficient to promote normal calcification in rats receiving sufficient fat-soluble vitamins in their diet.

When growth had ceased for 10–14 days, the diet was supplemented with daily amounts of 3, 5, 7, 10, 15 and sometimes 20 cc. of the milk, in order to find the dose necessary to restore a normal rate of growth. Three to four rats were used for each dose. Twenty or more rats were used in determining the value of each sample, and individuals from different litters were distributed as evenly as possible over the experiment, with controls receiving no milk. Milk feeding was continued for 35 days, and the standard of normal growth adopted was an average of 10–12 g. increase in weight per week during the experimental period. The mean between the dose that secured this growth and the one below it, was considered to contain one vitamin unit.

Use of egg white as the sole source of nitrogen in these experiments.

Boas [1924] has shewn that when cooked egg white is used as the sole source of protein, a loss of hair and declining weight occur in quickly growing rats, after 4–6 weeks.

For the first three months of my experiment the basal diet appeared to be perfectly satisfactory, the milk-fed animals had normal coats, whilst those of the controls shewed nothing more than the staring condition associated with vitamin A deficiency. There was no definite baldness.

In later trials the condition of baldness described by Boas occurred in some, but by no means all, of the rats during the preliminary period of feeding on the basal diet. This condition persisted in some of the controls, but was cured in the milk fed animals. The small amounts of caseinogen and lactalbumin given with the milk apparently supplied the protein deficiency. An analysis of bone made by rats of the same family with and without this coat condition, shewed that it did not affect the deposition of calcium in the bones. It was therefore decided to continue the use of egg white throughout

the experiment, as it was of the utmost importance to employ a rigid technique for the biological tests. The only variable factors in this experiment are the diet and light conditions of the cow, and the results are only to be interpreted by comparison with one another.

2. *Investigation of the anti-rachitic value of the milk.*

Whilst the titration of the growth-promoting power of the milk presents little difficulty, and the results are sufficiently accurate to permit of graphic representation, the anti-rachitic value cannot be determined with the same quantitative accuracy. It is the experience of all experimenters with rats, that when a group of animals is submitted to the same conditions, post-mortem signs of rickets do not occur consistently in each member of the group, unless the animal is, at the same time, starved of phosphorus, as is the case when McCollum's basal diet No. 3143 is employed.

With the basal diet described above, which contains an adequate supply of Ca and P, only a proportion of the control rats manifest post-mortem signs of rickets such as enlarged costochondral junctions, diminished rigidity of the skeleton, deformity of the chest and spontaneous fractures of the ribs. Taking all the control animals observed over 18 months, the proportion was 50 % by post-mortem, 60 % by histological diagnosis. When rickets ensued, consequently, it did not fall equally on all animals of a group. Sometimes all, but more often only 1, 2 or 3 of the 4 receiving a particular dose of milk would display obvious signs of rickets, whereas the others appeared to have more or less normal skeletons.

In view of this variation in the incidence of rickets, the post-mortem observations from period II onwards were supplemented in three ways. *Firstly*, a histological examination of the rib-junctions was made to confirm post-mortem observations. Two ribs from each animal, fixed in normal saline solution containing 10 % formalin for 48 hours, were partially decalcified in Müller's fluid, embedded in paraffin, sectioned and stained with haematoxylin and eosin. *Secondly*, the degree of calcification which had taken place in the five weeks' period of observation under the influence of the different rations of the various milk samples was determined, and compared with the deposition of calcium salts in the control animals which had received the basal diet only. The calcium content of the bones of both hind legs (femur, tibia and fibula) was estimated by Aron's method [Aron and Sebauer, 1910]. *Thirdly*, a determination was made of the minimal daily dose of a particular sample of milk required to prevent any histological manifestation of rickets in young rats fed upon McCollum's rickets-producing diet No. 3143[1]. Rats aged three to four weeks, when fed upon this diet without the addition of cod-liver oil, or other animal fat, invariably develop severe rickets. Using this method, it was possible to assign to each sample the possession of a

[1] Wheat 33 %, maize 33 %, gelatin 15 %, wheat gluten 15 %, NaCl 1 %, $CaCO_3$ 3 %.

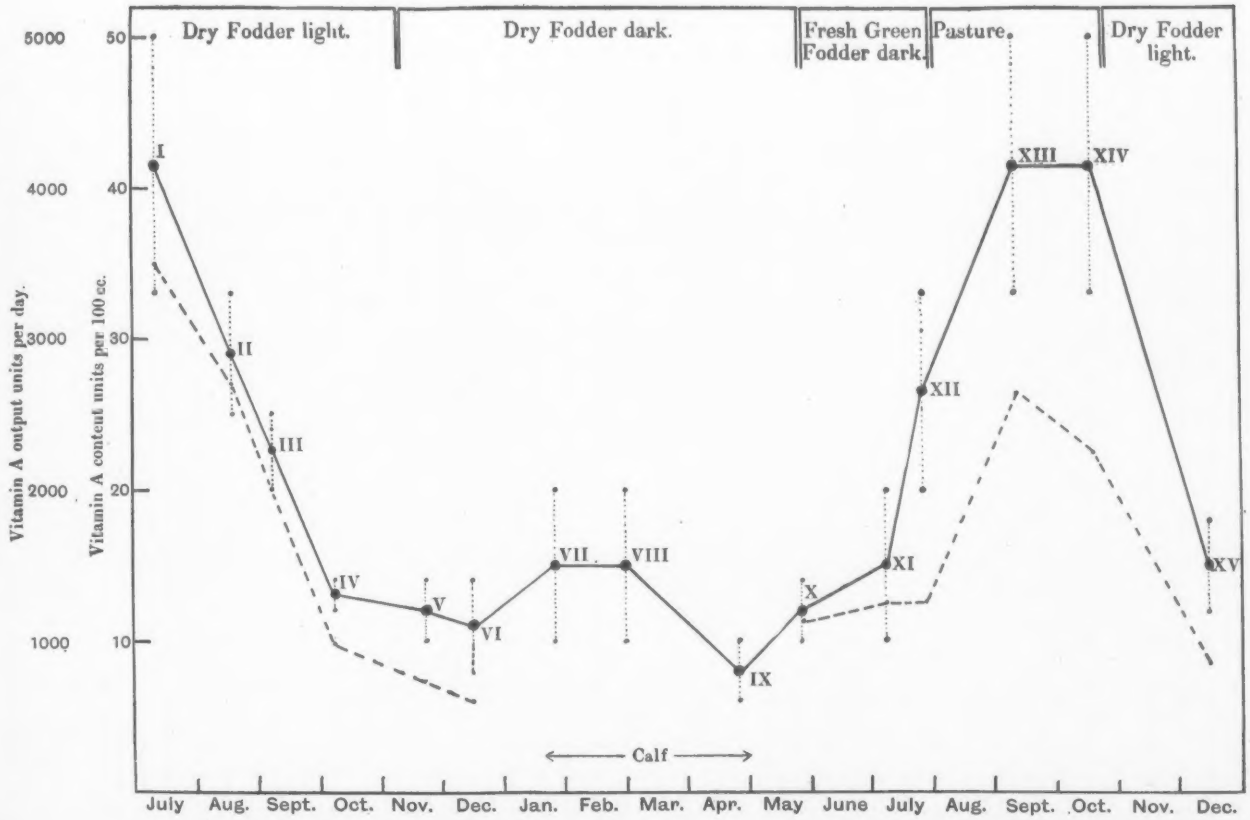

Fig. 1. Vitamin A (growth-promoting) value of milk expressed as units per 100 cc. Samples I–XV •——•——•——•
Total Vitamin output of the cow in units per day ·····

certain number of "anti-rachitic units," that is, the method was roughly quantitative. Young rats weighing 47–55 g. were fed on McCollum's 3143 diet supplemented with different rations of the various milks for 28 days and compared with control animals receiving no milk.

The employment of McCollum's diet is a very severe test for the anti-rachitic factor. Its content of phosphorus is inadequate, and this is a severe handicap to the process of calcification. On the other hand the estimation of the amount of calcium in the bones is a more sensitive indication of the disturbance of calcification and can be employed with a basal diet in which the amount of salts is adequate.

IV. Influence of diet and light upon the growth-promoting value of the milk.

In order to obtain comparable values for the growth-promoting value of the various samples of milk an arbitrary standard was fixed. This standard was the dose that restored a growth of 10 to 12 g. on an average per week in rats whose weight had become stationary on a diet deficient in fat-soluble vitamins.

This fixing of such an arbitrary end-point is open to criticism. The most one can say about its accuracy is that one particular dose was sufficient, whilst the one below was insufficient, to restore a certain rate of growth. The end-point, in reality, lies between the two, and is more accurately expressed in a graph showing the mean between two limits. This method of representation has been adopted in Fig. 1 and the two limits are indicated by the vertical lines. Tests were made with 15 samples of milk, taken at intervals over the five periods described at the beginning of the paper; the results showed that the limits from which the end-point was calculated varied according to the spacing of the doses, as shown in the following table:

Date	Period	Sample	Growth-promoting, end-point between	Growth units in 100 cc.
July 1922	I	I	2– 3 cc.	50–33
Aug.	„	II	3– 4	33–25
Sept.	„	III	4– 5	25–20
Oct.	„	IV	7– 8	14–12
Nov.	II	V	7–10	14–10
Dec.	„	VI	7 (–10)	14 (–10)
Jan. 1923	„	VII	5–10	20–10
Feb.	„	VIII	5–10	20–10
April	„	IX	10–15	10– 6
May	„	X	7–10	14–10
July	III	XI	5–10	20–10
July	„	XII	3– 5	33–20
Sept.	IV	XIII	2– 3	33–50
Oct.	„	XIV	2– 3	33–50
Dec.	V	XV	6– 8	18–12

The continuous line in Fig. 1 represents the number of vitamin units per 100 cc. in the samples tested; the broken line shows the total output in vitamin units per day reckoned from the total milk excretion of the cow.

From February–April, 1923, the cow was suckling her calf, so that during this period, as indicated in the graph, the total milk excretion was not measured.

It is evident that the growth-promoting power of the milk is controlled by the *diet of the cow*. In period I when conditions of illumination were unchanged, a drop from 41 to 13 units per 100 cc. was obtained by changing from a diet of pasture to one of dry food deficient in fat-soluble vitamins; and a rise in period III from 12 to 26 units by the substitution of fresh green food for dry fodder.

It is also evident that the effect of diet is *not immediate*. It took three months for the growth-promoting power of the milk to fall from 41 to 13 units per 100 cc. in period I. At the beginning of the experiment the animal was in good condition. She had previously been out at pasture for three months, and during this time had received in addition a daily ration of hay and oil cake. A preliminary test showed her to be giving milk of high vitamin A value (41 units per 100 cc.), and she had, in all probability, a considerable store of vitamin A in her tissues. It is, therefore, not surprising that it should take time to deplete this store. It is also possible that *three months was too short a time to produce the maximum effect*, and that the further small drop obtained during period II might have occurred, had the cow been kept out of doors, on the same diet, a couple of months longer.

The calf was born on January 27th, 1923. The last ante-partum sample of milk tested (sample VI) was of low vitamin A value[1], the first two post-partum samples (VII and VIII) showed a slightly improved value. Whilst this may be only a variation, within normal limits, of the growth-promoting power of the milk, there is also the possibility of there being an ante-partum storage, and subsequent post-partum liberation of the growth factor, for the needs of the calf.

The observations afford *no evidence as to whether the degree of illumination of the cow influences the growth-value of the milk.*

(*a*) The small diminution from 13 to 8 vitamin A units per 100 cc. which occurred in darkness following three months on dry fodder out of doors and (*b*) the striking improvement from 26 to 41 units per 100 cc. when pasture feeding was substituted for grass feeding in the dark, both suggest a beneficial influence of light. Both, however, might have happened had the periods been extended during which illumination was kept constant.

V. The influence of diet and light upon the anti-rachitic properties of the milk.

(*a*) *Evidence from post-mortem observations and histological diagnosis on rats receiving the basal diet supplemented by different doses of the milks.*

At the beginning of the experiment in period I, when the cow was fed on cereals and oats and straw, and kept out of doors, the post-mortem appear-

[1] The scale of doses used for testing this sample did not go above 10 cc., which was inadequate as indicated by dotted line in graph.

ance of the rats receiving milk was not sufficiently striking to warrant a histological examination being undertaken. Three out of twelve control animals showed post-mortem evidence of rickets, but none of the rats receiving 2 cc. of milk or upwards showed macroscopic evidence of rickets.

A striking change was observed in the post-mortem appearances of the rats receiving milk from sample VI (period II, Protocol *A*). Out of 15 animals in this group, receiving doses of milk ranging from 3–10 cc., 11 showed post-mortem, and 15 histological, evidence of rickets—2 out of 3 control animals in this group were also rachitic, the other showed an extreme degree of osteoporosis. It is obvious, therefore, that a daily dose of 10 cc. of this milk was not protective.

As a result of this observation it was resolved in future to supplement post-mortem observations in all animals by histological examination of costochondral junctions and determinations of the calcium content of the bones.

Six samples of milk were tested during period II, whilst the cow was on dry fodder but kept in complete darkness, and during this time the rats receiving both 3 and 5 cc. doses of milk showed an even higher percentage of rickets than the controls of this period, and many receiving up to 10 and 15 cc. were also rachitic.

During the following two months, period III, when the cow was still in the dark but fed on fresh grass and clover, the rats receiving 3 cc. doses still showed a higher percentage of rickets than the controls of this period, whilst the percentage occurrence of rickets in those receiving 5 cc. doses was slightly lower. Two out of six rats receiving 10 cc. of milk during this period showed evidence of slight rickets histologically.

The milk obtained after six weeks of pasture feeding (period IV) showed a marked change. Out of 40 rats receiving doses varying from 1 to 15 cc. daily, only one showed evidence of rickets at post-mortem examination. This was one of eight animals which received a 1 cc. dose of milk and proved on histological examination to show evidence of osteoporosis and not of rickets. Four out of eight control animals in this series showed histological evidence of rickets.

On reverting to the diet of dry fodder for two months (period V), the cow being still out of doors, the milk retained its high anti-rachitic value. None of the rats receiving 3 cc. doses and upwards showed any signs of rickets, while two out of the four control animals showed histological evidence of rickets.

An analysis of the incidence of rickets among the rats receiving the milk corresponding to the different periods of the cow's history tells the following story:

(*a*) When the cow was kept out of doors in the light, whether on dry fodder or pasture, none of the rats receiving 3 cc. doses and upwards showed histological evidence of rickets, which occurred in 50 % of the control animals.

(*b*) The highest incidence of rickets in milk-fed rats occurred with milk obtained during the period of dry feeding in the dark. Animals receiving 3 and 5 cc. doses of this milk daily showed an even higher percentage of rickets than the control animals. Doses of 15 cc. and upwards were usually needed to prevent rickets.

(*c*) The incidence of rickets was still high in animals receiving the milk obtained during the period of grass feeding in the dark[1].

(*b*) *Evidence from the calcium content of the bones of the above animals.*

The calcium determinations tell nearly the same story as the post-mortem and histological observations. The results of these are displayed in Fig. 2, which shows the percentage of calcium in the bones of rats used for titrations of milk samples V–XV plotted against the daily quantity of milk fed.

During the period of dry feeding in the dark (period II) 3 cc. of milk daily caused no improvement in calcification as compared with that of the controls; there is some, but no very great improvement with 5 cc. (Fig. 2 and Protocols *A* and *B*). Sample VI, already referred to as being low in its growth-promoting and anti-rachitic power, is seen to possess the lowest calcium depositing power of the samples belonging to period II (Fig. 2 and Protocol *A*). 10 cc. of this milk gave about the same calcification as 5 cc. of the previous and two later samples. This sample, as already stated, was the last one tested before the birth of the calf. This sample of milk, then, besides being of low anti-rachitic and growth-promoting value, showed also the lowest calcium depositing value of any milk used throughout the experiment.

With green feeding in the dark (period III, Protocol *C*) there is some improvement in the calcifying power of the milk obtained; most obvious in the case of the low doses of sample XII, a milk taken after two months of this feeding. This was not accompanied by a decrease in the incidence of rickets as shown by histological examination, a fact possibly connected with the deprivation of light, suffered by the cow during this period.

The best calcification, however, was obtained with the rats receiving milks of period IV, when the cow was pasture fed. Of the two samples tested, the first (sample XIII) caused better calcification with a 5 cc. dose (20·4 % Ca) than had been obtained with a 20 cc. dose of any previous sample. For the testing of this sample, however (Protocol *D*), only two families of rats were used, and only two rats on each dose and in this case a higher average percentage calcification was obtained in the control animals than was the case in the antecedent groups. The animals used for this test were possibly at a higher level as regards their bodily store of fat-soluble vitamins, than those used in previous tests, as is seen by the increased growth and calcification in the controls. This observation was made from July to September, during the time when laboratory light conditions were at a maximum. The animals were

[1] The histological picture in nearly all the milk-fed animals showed some degree of osteoporosis.

never exposed to direct sunlight, the windows being kept closed. In spite of this fact, however, the control rats showed no histological evidence of rickets, only a moderate degree of osteoporosis. The second sample (Protocol *D* 1) of pasture milk, tested a month later, showed a similar high calcium-depositing value and in this case the control animals were comparable to those of periods II and III, inasmuch as they grew badly, had badly calcified bones, and all showed post-mortem and histological evidence of rickets. *All the animals receiving milk, from* 1 *cc. and upwards, in this period, when the cow was on pasture, showed an improved calcium content of bone as compared with the controls. The contrast was well-marked with the low doses.*

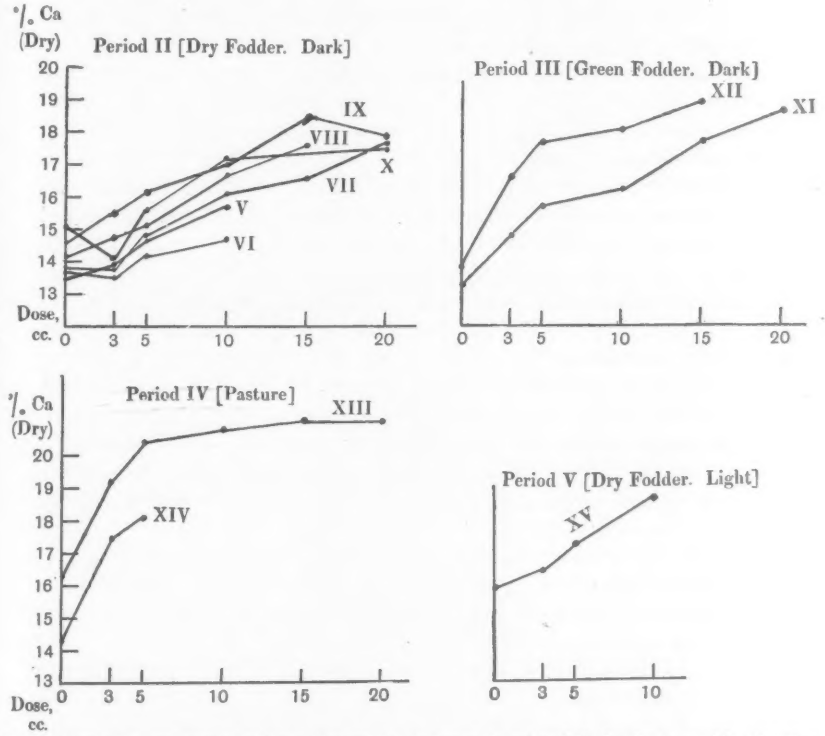

Fig. 2. Average percentage of calcium (dry weight) in bones, femur and fibula of rats used for titration of milk samples V–XV (see Protocols *A* to *E*). Time of experiment 35 days. 3–4 rats were used for titration of each dose.

The calcium content of the bones of the rats used to investigate the milk of period V (dry feeding in the light, Fig. 2 and Protocol *E*) shows a drop in the case of the animals receiving low doses compared with those receiving the milk from the cow when on pasture.

(c) *Evidence from experiments using McCollum's rickets-producing diet No.* 3143.

Samples X, XII, XIV and XV, taken at the end of periods II, III, IV and V respectively, were used to determine the anti-rachitic power of the milks. Each of these samples was titrated over a family of eight or nine rats receiving doses of 2, 5, 7, 10, 15 and 20 cc., with two controls receiving no milk. As far as possible animals of the same sex and initial weight received the same dose in each of the four samples. The animals were fed for 28 days on McCollum's 3143 diet, with the addition of doses of the various milks. In every case a histological examination of the rib-junctions, and calcium determination of the leg bones (femur, tibia and fibula) were made.

The protective dose of milk was taken to be the one received by rats giving an approximately normal histological picture.

All the *control animals* in the four groups showed gross and histological evidence of severe rickets. The *macroscopic findings* were marked enlargement of the costochondral junctions, angulation and spontaneous fractures of the ribs, enlarged radio-ulnar epiphyses, with marked diminished rigidity and deformity of the chests. *Histologically* there was very imperfect calcification, excess of osteoid tissues, both cortical and peri-trabecular, angulation of ribs, increase in width and disorganisation of the zone of hypertrophic cartilage cells, and disorganisation of the zone of provisional calcification. The milk-fed animals showed evidence of rickets that varied with the quantity and quality of the different milks.

The results are seen from Tables I–IV and are summarised in Table V and illustrated by Figs. 3–7 (Plates II and III).

1. *At the end of period II* (dry feeding in the dark) (Table I and Fig. 7) 15 cc. of sample X was barely protective. Rat 599 receiving 15 cc. of this milk showed a histological picture of slight rickets. Rat 600 receiving 15–20 cc. showed an approximately normal picture.

2. *At the end of period III* (green feeding in the dark) (Table II and Fig. 6) a normal histological picture was obtained in rats that had received 20 cc. daily, but all the other milk-fed animals showed a histological picture of severe or pronounced rickets.

3. *At the end of period IV* (pasture feeding) (Table III and Figs. 3, 4 and 5) there was a marked improvement in the histological picture obtained with rats that had received 2 cc. daily, and a normal picture with those that had received 5 cc. The same result was obtained with a second family of rats tested with low doses only (1–5 cc.).

4. *At the end of period V* (dry feeding in the light) (Table IV) the quantity of milk required to produce a normal histological picture in rats on McCollum's diet had increased to 10 cc. The test was made with two litters.

Calcium-content of the bones of these animals. In every group the animals showed an increasing deposition of calcium with increasing doses of milk.

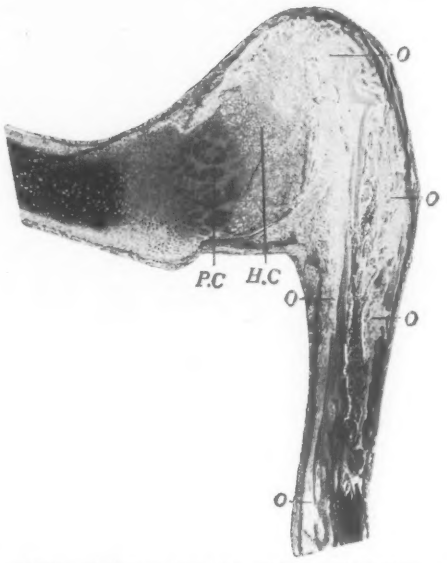

Fig. 3. Rib-junction, rat 796; 28 days on basal diet without milk addition. Severe rickets, rib-junction bent at an angle, structure disorganised: wide zone of hypertrophic cartilage (*HC*); excess of osteoid tissue (*O*); zone of proliferating cartilage (*PC*).

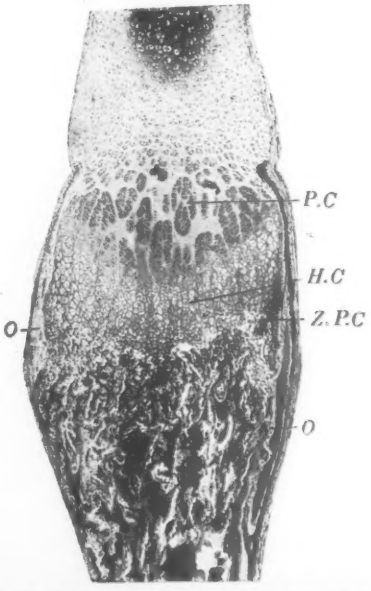

Fig. 4. Rib-junction, rat 797; 28 days on basal diet with addition of 2 cc. daily of milk sample XIV (period IV, pasture-feeding). Slight rickets. Zone of hypertrophic cartilage (*HC*) still wide, but reduced in width in comparison with rat 796: much less osteoid tissue (*O*).

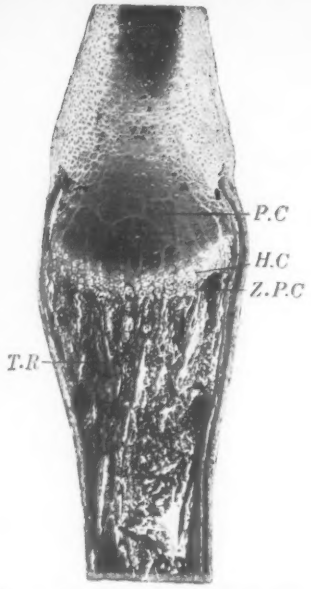

Fig. 5. Rib-junction, rat 801; 28 days on basal diet with addition of 5 cc. daily of milk sample XIV (period IV. pasture feeding). Structure nearly normal, well-marked zone of provisional calcification (*ZPC*); and well-ossified bony trabeculae (*TR*).

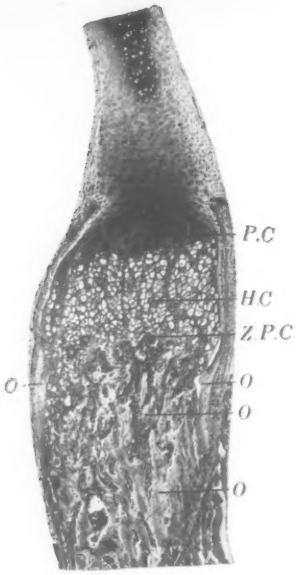

Fig. 6. Rib-junction, rat 724; 28 days on basal diet with addition of 5 cc. daily of milk sample XII (period III fresh green food in a dark stall). Pronounced rickets. Wide zone of hypertrophic cartilage (*HC*) with very deficient calcification in zone of provisional calcification (*ZPC*); excess of osteoid tissue (*O*).

Fig. 7. Rib-junction, rat 596; 28 days on basal diet with addition of 5 cc. daily of milk sample X (period II, dry-fodder in a dark stall). Pronounced rickets. Structure much disorganised, wide zone of hypertrophic cartilage (*HC*), much osteoid tissue (*O*); zone of provisional calcification remains almost uncalcified.

Figs. 2–7. Basal diet = McCollum's rickets producing 2143 diet.

ANTI-RACHITIC POWER OF THE MILKS. RATS ON McCOLLUM'S 3143 DIET. MILK FEEDING 28 DAYS.

Table I. *Period II.* (*Dry fodder, dark.*) *Sample X.*

Rat	Sex	Family	Dose	Initial weight	Final weight	Post-mortem evidence of rickets	In bones % H_2O	In bones % Ca wet	In bones % Ca dry	Histological diagnosis
593	♂	A	No milk	49	93	+ + +	63·2	4·2	11·4	Severe rickets
594	♀	„	„	53	83	+ + +	63·1	3·8	10·2	„
					Av. no milk:		**63·1**	**4·0**	**10·8**	
			cc.							
595	♂	„	2	54	95	+ +	57·0	6·4	14·7	Pronounced rickets
596	♀	„	5	49	90	+ +	55·6	6·9	15·6	„
597	♂	„	7	52	120	+ +	55·0	7·5	16·8	„
598	♀	„	10	47	98	+ +	Estimation lost			„
599	♂	„	15	48	142	+	51·3	8·8	18·9	Slight rickets
600	♀	„	*15–20	48	110	?	48·8	9·9	19·5	Nearly normal

Table II. *Period III.* (*Green fodder, dark.*) *Sample XII.*

Rat	Sex	Family	Dose	Initial weight	Final weight	Post-mortem evidence of rickets	In bones % H_2O	In bones % Ca wet	In bones % Ca dry	Histological diagnosis
721	♂	B	No milk	47	67	+ + +	62·4	4·3	11·3	Severe rickets
722	♀	„	„	53	82	+ + +	61·0	4·1	10·5	„
					Av. no Milk:		**61·7**	**4·2**	**10·9**	
			cc.							
723	♂	„	2	52	106	+ + +	61·6	4·3	11·3	„
724	♀	„	5	45	84	+ + +	56·4	6·6	15·1	Pronounced rickets
725	♂	„	7	48	102	+ + +	59·4	5·8	14·3	„
726	♀	„	6–10	37	68	+ + +	60·8	4·7	12·1	„
727	♂	„	12–15	47	110	+ +	59·1	5·8	14·2	„
728	♂	„	*20	55	138	?	59·1	8·6	17·8	Nearly normal

* Protective dose.

There was no definite correspondence, however, between the proportion of calcium in the bones and the histological picture.

The possible influence of increased phosphorus given with the milks. The possibility that the curative effect of feeding the various milks is partly due to the ingestion of phosphorus, apart from the presence of the organic anti-rachitic factor, has not been lost sight of. An analysis of the four samples of milk used in these experiments, kindly made by Miss Boas, gave the following results:

Sample	% P	% Ca
X	0·1039	0·123
XII	0·0803	0·116
XIV	0·0901	0·118
XV	0·0913	0·1323

The ratio of Ca to P in McCollum's 3143 diet is 4 : 1. The extra Ca and P given in a daily addition of 2 cc. of milk alters it to 3·8 : 1, and addition of 5 cc. of milk to 3·5 : 1. With the higher doses 10, 15 and 20 cc. of milk

Table III. *Period IV. Pasture (July to October). Sample XIV.*

Rat	Sex	Family	Dose	Initial weight	Final weight	Post-mortem evidence of rickets	In bones % H_2O	Ca wet	Ca dry	Histological diagnosis
795	♀	C	No milk	50	70	+ + +	61·1	5·4	14·0	Severe rickets
796	♂	„	„	52	61	+ + +	62·7	4·5	12·1	„
						Av. no milk:	**61·9**	**4·9**	**13·1**	
			cc.							
797	♂	„	2	50	83	+	57·2	7·1	16·9	Slight to moderate rickets, healing
801	♀	„	*5	49	90	?	49·3	8·8	17·5	Nearly normal
799	♂	„	7	51	92	?	53·4	8·4	18·1	„
800	♀	„	10	47	107	?	50·9	9·2	19·0	Slight osteoporosis
802	♀	„	20	48	107	—	51·4	9·4	19·5	Nearly normal
Second family. Low doses only:										
811	♂	D	No milk	49	69	+ + +	58·7	5·8	14·2	Severe rickets
812	♀	„	„	53	82	+ + +	58·7	5·5	13·4	„
						Av. no milk:	**58·7**	**5·6**	**13·8**	
			cc.							
818	♀	„	1	47	72	+ + +	55·8	6·3	14·4	„
819	♀	„	1	45	65	+ +	53·5	7·2	15·5	Pronounced rickets
						Av. 1 cc.:	**54·6**	**6·7**	**14·9**	
817	♂	„	2	48	86	?	53·9	7·1	15·6	Slight rickets
815	♀	„	3	48	83	?	52·9	7·5	16·1	Nearly normal
816	♀	„	3	46	83	?	54·5	7·1	15·7	Slight rickets
						Av. 3 cc.:	**53·7**	**7·3**	**15·9**	
813	♀	„	*5	49	93	?	55·4	7·1	15·9	Nearly normal

Table IV. *Period V. (Dry fodder, light.) October to December. Sample XV.*

Rat	Sex	Family	Dose	Initial weight	Final weight	Post-mortem evidence of rickets	In bones % H_2O	Ca wet	Ca dry	Histological diagnosis
914	♂	E	No milk	53	85	+ +	59·9	5·7	14·2	Pronounced rickets
915	♀	„	„	51	75	+ + +	62·1	4·7	12·4	Severe rickets
						Av. no milk:	**61·0**	**5·2**	**13·3**	
			cc.							
916	♂	„	2	52	85	+ +	59·2	5·8	14·2	„
917	♀	„	5	50	107	+	56·0	7·2	16·3	„
918	♀	„	7	52	104	+	55·6	7·2	16·3	Pronounced rickets
919	♀	„	*10	51	110	?	50·6	9·2	18·7	Nearly normal

* Protective dose.

Table V. *Rickets-preventing value of milk samples.*

No. of sample	Description	Protective dose against rickets for rats on McCollum's 3143 diet, between
X	After 6 months on dry fodder given in dark stall	15 cc. – and 20 cc. +
XII	After 2 months on fresh green fodder in dark stall	12 to 15 cc. – and 20 cc. +
XIV	After 3 months of pasture-feeding (July–October)	2 cc. – and 5 cc. +
XV	After 2 months on dry fodder given out of doors (Oct.–Dec.)	7 cc. – and 10 cc. +

the change is greater and the ratio becomes 2·5–2·7 : 1, and it is possible that this proportional increase in the amount of P may contribute to the preventive effect of certain high doses of milk.

The results of these experiments, however, have been interpreted after comparison of the effect of doses of similar size, the only variable factor being the sample of milk tested. They are important in so far as they show a positive anti-rachitic effect of *small doses* (1–5 *cc. daily*) *of milk from the cow on pasture feeding*. With these doses the added phosphorus is in all probability negligible, and the result obtained indicates a high anti-rachitic potency of the milk.

The results obtained with the higher doses cannot be interpreted to indicate only anti-rachitic potency, as the extra P given with the milk probably contributes to the result.

The Calf.

The calf (♀) was born on January 27th, 1923, during the period when the cow was in the dark and fed on cereals, roots and straw. She weighed 70 lbs. at birth and was apparently normal and healthy. She was kept with the mother in the dark and fed exclusively on the mother's milk. Three months later she was killed. By this time she was well grown and weighed 300 lbs.

Post-mortem examination showed nutrition good, subcutaneous and body fat plentiful: bones of skull and limbs hard: no enlargement of costochondral junctions: viscera normal.

A *calcium analysis* was made of the 6th rib, a length equal to 2 cm. cut at equal distances from the costo-vertebral margin on both sides, and gave the following result.

	Analysis of rib		
	% H_2O	% Ca wet	% Ca dry
6th rib—right side	28·7	15·8	22·2
,, —left side	29·0	15·8	22·2

A *histological examination* of the costochondral junction showed no excess of osteoid tissue, no increase in width of the zone of hypertrophic cartilage cells, or disorganisation of this area, a normal area of provisional calcification, and bone trabeculae regularly arranged and well calcified. Very slight osteoporosis.

Results from observations on the calf. The milk used for feeding the calf was that of period II in which the growth-promoting and anti-rachitic value, as tested on rats, had reached a minimum. In spite of this she grew normally and showed no signs indicative of rickets.

The mother, during the first three months of pregnancy, had been on pasture, receiving in addition a daily ration of hay and oil cake. Previously to this she was known to have had several healthy calves, and to be a "heavy milker." She must, therefore, have been in excellent condition at the beginning of the experiment, with a maximum storage of growth and anti-rachitic factors.

The calf, then, lived for the first three months of intra-uterine life under optimal conditions of nutrition, the mother receiving abundance of light, and a diet rich in the fat-soluble vitamins. A deprivation of fat-soluble vitamins for six months and of light for two months did not apparently affect the calf, which was probably, *in utero*, drawing upon the mother's reserve of growth and anti-rachitic factors during this time. In the lactation period of three months that followed, the milk proved adequate for the needs of the animal.

Rickets is not a common disease amongst calves, but there is some evidence in support of the view that the onset of the disease may take place after three months of age if the period of lactation is prolonged.

McCandlish [1923], investigating milk as an exclusive diet for calves, found that it would not support life after the first three or four months. Two bull calves fed exclusively on milk, did well for the first 13 weeks, but after that declined and died within six months. In these animals there was post-mortem evidence of softening of the bones, and spontaneous fractures of the ribs. In a later paper he investigated the effect of the addition of mixed grain and alfalfa hay to the milk ration. A calf fed on the diet of milk and mixed grain ultimately developed "stiff and swollen leg joints with knees bowed outward and forward." The photograph of this animal gives an almost typical picture of rickets, although, as this animal was subsequently cured by the addition of alfalfa hay it did not come to post-mortem examination.

It is possible that had the milk feeding been continued longer with unchanged feeding and management of the mother a condition resembling rickets might have been induced in the calf, born during the present experiment. The reason why rickets is not a disease of common occurrence amongst calves may be that at three months of age the diet of a calf is normally changed from one of milk to one of grain, hay, pasture, roots, etc. Milk is of low anti-rachitic value as compared with green fodder and hay, and the reinforcement of the calf's diet with food of high anti-rachitic value may serve to tide the animal over a period of susceptibility.

A sample of colostrum milk, taken 18 hours after calving, was titrated for 35 days with a single family of six male rats. The table shows *a definite increase in the growth-promoting power of low doses of colostrum, compared with that of the preceding milk, sample VI, taken during period II* (compare Protocol *A*).

With the exception of rat 428, which showed a slightly better calcification than the control animal of this series, this increased growth was not accompanied with increased calcification in rats receiving doses up to 7 cc. The incidence of rickets in this group of rats was as high as in those receiving previous samples of the milk of this period when the cow was in the dark on dry feeding.

Colostrum milk. 18 *hours after calving.*

Rat	Sex	Dose	Initial weight	Final weight	Post-mortem evidence of rickets	In bones % H_2O	Ca wet	Ca dry	Histological diagnosis
422	♂	No dose	53	74	+	43·4	8·0	15·5	Osteoporosis + mod. rickets
428	♂	3 cc.	58	119	–	46·3	8·9	16·6	Osteoporosis
426	♂	5	49	101	+	46·1	8·3	15·3	Osteoporosis + mod. rickets
427	♂	7	52	104	+	46·4	7·9	14·8	,, ,,
423	♂	10	54	122	–	45·4	9·2	17·1	Osteoporosis
425	♂	10	57	129	–	46·4	9·4	17·5	Osteoporosis + slight rickets
					Av. 10 cc.	45·9	9·3	17·3	

Variations in the amount of sunshine during periods I, IV and V.

To control the diet of a cow is a simple matter, but light conditions vary with the time of year and cannot be controlled or standardised. Records of the amount of sunshine (in hours per day) taken at Rothamsted Meteorological Station, Herts., show that in period I, July–November, 1922, there was a decreasing amount of sunshine ranging from an average of 4·8 hours per day in July to 1·9 hours per day in November. The maximum amount of sunshine recorded in this year was 8·2 hours per day in May and June, before the beginning of the experiment. The amount of sunshine per day impinging on the cow during period I, which averaged 4·2 hours per day from July to October, was decreasing all the time and never approached the maximum for the year.

In period IV the records show an average of 7·3 hours of sunshine per day, which was *the maximum for the year.*

In period V the records compare with those of period I, that is to say, the light was decreasing and averaged 3·4 hours per day in November and 1·4 hours per day in December.

These observations show that whereas the intensity of sunshine during the two periods of dry fodder in the light (periods I and V) is roughly comparable, neither can be compared with that prevailing during the period of pasture feeding (period IV). It is possible that before light can be effective in raising the anti-rachitic value of milk, it must reach a certain level of intensity. Further experiments of dry feeding *during maximum conditions of sunshine* are being attempted, in order to elucidate this point.

Summary of Section V.

The milk, at the beginning of the experiment, when the cow had been out at pasture, gave evidence of anti-rachitic properties in doses of 2 cc. and upwards. A fall in the anti-rachitic value was not recognisable until the cow had been fed on a diet deficient in fat-soluble vitamins for $4\frac{1}{2}$ months, by which time the growth-promoting (vitamin A) value of the milk had reached a minimum.

On changing the diet to fresh grass the cow being kept still in the dark, the anti-rachitic value of the milk improved slightly, but this improvement was not great enough to be detected with McCollum's 3143 rickets-producing diet.

On letting the cow out to grass, the milk regained its original value, *and this improvement was also evident by the severe test with McCollum's* 3143 *rickets-producing diet.* Two months after dry food had been again substituted for pasture, the cow remaining out of doors, the milk still contained anti-rachitic properties to a considerable degree.

VI. General Discussion of Results.

There are three possible theories in explanation of the results obtained in these experiments:

1. *That the growth-promoting factor in milk is controlled by the diet of the cow, whilst its anti-rachitic value depends upon the amount of light impinging on the animal.* This theory gains some support from the fact that during both periods I and V, in which the cow was fed on dry fodder out of doors, the growth value of the milk decreased almost to a minimum, whilst the anti-rachitic value remained fairly high, in spite of the fact that the intensity of light was on the decline. Conversely, during periods II and III, when the cow was kept in the dark stall on dry fodder, and fresh green food, respectively, the percentage incidence of rickets in the milk-fed animals was greater than during the three periods when she was out of doors. This low anti-rachitic value appeared to hold, notwithstanding the increased growth-promoting value observed in the colostrum milk yielded during period II and in the milks tested after the cow's diet had been changed to fresh green food in period III.

2. *That both growth-promoting and anti-rachitic factors in milk are influenced only by the diet of the cow.* A fall in the anti-rachitic value of the milk occurred after a $4\frac{1}{2}$ months' deficiency of fat-soluble vitamins in the diet of the cow. The growth-promoting value was reduced almost to a minimum after three months' deficient feeding. Furthermore, both factors in the milk were at their maximum and minimum values together. It is possible that both are controlled by diet, but that it takes longer to drain a cow of the anti-rachitic than of the growth-promoting principle.

If this were so it should be possible to reduce both factors to a minimum by dry feeding out of doors, and raise them to a maximum by green feeding in the dark. The periods employed in these observations were not long enough to permit of this conclusion.

3. *That the diet of the cow has the main effect on both factors, whilst light controls to some extent the anti-rachitic value of the milk.* It is evident that diet affects both factors, but how far the anti-rachitic value of milk is dependent on the amount of sunlight impinging on the cow is not apparent, though there are indications that it has a part, possibly a subsidiary one, to play. As long as the cow remained in the light, even light of a fairly low intensity (period I, July–November, and period V, October–December), the anti-rachitic value of the milk remained at a higher level than that reached when the animal was in the dark. Also no definite fall was observed in the anti-rachitic value of the milk until the cow had been, for some weeks, in the dark. During the comparatively short period in which she received fresh

grass fodder in the dark the anti-rachitic value of the milk rose slightly, as judged by the calcium content of the bones of the animals fed upon it and might have risen more had this period been prolonged.

A maximum level of anti-rachitic potency, however, was not reached until the cow was placed under conditions of maximal light and optimal diet, and it is conceivable that, in the absence of sunlight, it would be impossible to raise the anti-rachitic value of the milk above a certain level.

In the absence of results from further experiments it is impossible to do more than draw broad conclusions. In the above experiments the variations in anti-rachitic value did not follow precisely those in (vitamin A) growth-promoting factor, although both were at their maximum and minimum together. Both are evidently influenced by diet. The discrepancy between them could be interpreted to mean, either that the anti-rachitic value of milk is influenced by the illumination of the cow more than the vitamin A value, or that it takes longer to exhaust the milk of the former active principle.

VII. Conclusions.

1. Milk from the same cow has been shown to vary within wide limits, as regards its anti-rachitic and growth-promoting power.

2. The diet of the cow appeared to be the main factor in determining the growth-promoting value of the milk. When the cow received a diet of fresh green grass the milk possessed a higher growth-promoting value than when she received dry fodder deficient in fat-soluble vitamins. This result was obtained irrespective of whether the cow was kept out of doors in sunlight or in a dark stall. The evidence as to whether light does, or does not, contribute, is not conclusive from these experiments.

3. The anti-rachitic value depends on the diet of the cow, and possibly also on the degree of illumination to which she is exposed. *Milk from a pasture fed cow has a definite and high anti-rachitic value*; the same animal when stall fed in the dark, yielded a milk much inferior in anti-rachitic properties.

4. A possible contributory cause of the seasonal incidence of rickets in children, may be the use of milks whose anti-rachitic value is higher in summer than in winter.

In conclusion, my thanks are due to Dr MacConkey and his staff at the Serum Department of the Lister Institute, Elstree, Herts., for their kind co-operation in carrying out the management of the cow, and much tedious work connected with this investigation. Without their co-operation the experiment could not have been undertaken. I also acknowledge with sincere pleasure my gratitude to Dr C. J. Martin for much valuable support and criticism during the course of the experiment, to Dr V. Korenchevsky and Dr H. Chick for technical advice, and to Miss M. Boas for kindly undertaking chemical analyses of the milks used. I wish also to thank Miss M. Rickards for assistance with section cutting, and G. Favell, for help in feeding and care of the animals.

PROTOCOLS *A–E*. SHOWING RESULTS OF TYPICAL ANALYSES OF MILKS FROM PERIODS I–V.

Protocol A. Period II. Sample VI. Dry fodder—dark.

Rat	Sex	Family	Dose	Initial weight	Final weight	Post-mortem evidence of rickets	In bones % H_2O	Ca wet	Ca dry	Histological diagnosis
415	♀	1	No milk	72	70	+ +	52·7	8·0	13·3	Osteoporosis + pronounced rickets
400	♂♀	2	,,	68	70	? +	53·0	8·5	14·0	Extreme osteoporosis
395	♂♀	3	,,	53	62	+ +	48·1	7·0	14·0	Osteoporosis + moderate rickets
					Av. no milk:		**50·1**	**7·7**	**13·7**	
			cc.							
401	♂	2	3	65	70	+ +	48·2	6·9	13·5	Osteoporosis + slight rickets
402	♂♀	2	3	56	68	+ +	52·7	6·0	12·7	Pronounced rickets
408	♂	1	3	56	93	+ +	53·7	6·6	14·3	Osteoporosis + slight rickets
					Av. 3 cc.:		**51·5**	**6·5**	**13·5**	
396	♀	3	5	52	69	+	41·4	7·4	14·7	Slight rickets
406	♂♀	2	5	74	80	+	46·7	6·8	12·9	,,
409	♂	1	5	74	93	+	49·3	7·2	14·1	,,
411	♂	1	5	66	106	+	49·0	7·7	15·2	Osteoporosis + moderate rickets
					Av. 5 cc.:		**46·6**	**7·3**	**14·2**	
397	♀	3	7	53	85	–	42·9	7·8	13·7	Moderate rickets
399	♀	3	7	63	95	+	44·9	8·3	14·9	Osteoporosis + slight rickets
412	♂	1	7	65	100	+	52·0	6·8	14·3	,, ,,
					Av. 7 cc.:		**46·6**	**7·6**	**14·3**	
398	♂	3	10	61	100	+	48·7	6·9	13·6	,, ,,
405	♀	2	10	58	88	+	48·1	7·9	15·4	Slight rickets
403	♂♀	2	10	71	94	–	45·5	6·9	13·5	Osteoporosis + slight rickets
414	♀	1	10	54	105	–	49·6	8·2	16·4	,, ,,
					Av. 10 cc.:		**47·4**	**7·6**	**14·7**	

Last ante-partum sample tested. Showing high incidence of rickets and low % calcium in bones.

Protocol B. End of Period II. Sample IX. Dry fodder—dark.

Rat	Sex	Family	Dose	Initial weight	Final weight	Post-mortem evidence of rickets	In bones % H_2O	Ca wet	Ca dry	Histological diagnosis
553	♂	1	No milk	55	113	+	48·6	7·6	15·1	Osteoporosis + pronounced rickets
536	♂♀	2	,,	57	117	+	51·2	7·1	14·6	Osteoporosis + moderate rickets
573	♂♀	3	,,	67	65	+	49·9	7·3	14·3	Osteoporosis + slight rickets
					Av. no milk:		**49·9**	**7·3**	**14·6**	
			cc.							
537	♀	2	3	92	113	+	45·0	9·3	16·9	Osteoporosis + pronounced rickets
554	♂♀	1	3	73	88	+	45·5	8·5	15·6	Osteoporosis + slight rickets
561	♂	1	3	73	95	+	44·1	7·9	14·2	,, ,,
572	♂	3	3	67	120	–	Estimation lost			,, ,,
					Av. 3 cc.:		**44·8**	**8·5**	**15·5**	
562	♀	1	5	60	91	–	45·4	8·4	15·5	Osteoporosis
563	♀	1	5	90	125	–	40·7	10·1	16·9	Osteoporosis + slight rickets
					Av. 5 cc.:		**43·0**	**9·2**	**16·2**	
541	♀	2	10	103	133	–	44·1	9·9	17·8	Osteoporosis
551	♂♀	1	10	87	127	+	47·5	8·5	16·2	Osteoporosis + slight rickets
555	♂	1	10	78	128	+	47·5	9·1	17·4	,, ,,
574	♂	3	10	63	117	+	46·9	8·8	16·7	Osteoporosis + pronounced rickets
					Av. 10 cc.		**46·5**	**9·0**	**17·0**	
543	♀	2	15	89	133	–	44·0	10·6	19·0	Slight osteoporosis
552	♂♀	1	15	95	138	–	41·1	10·8	18·3	Osteoporosis + slight rickets
560	♂	1	15	87	142	–	47·9	9·4	18·2	,, ,,
					Av. 15 cc.		**44·3**	**10·2**	**18·5**	
564	♀	1	20	85	144	–	41·1	10·1	18·0	Slight osteoporosis
565	♂♀	2	20	85	158	–	40·8	10·1	17·1	,,
550	♂	1	20	94	167	–	46·8	9·5	17·9	Osteoporosis
					Av. 20 cc.:		**40·9**	**10·1**	**17·5**	

Showing high incidence of rickets.

Protocol C. Period III. Sample XI. Fresh green fodder—dark.

Rat	Sex	Family	Dose	Initial weight	Final weight	Post-mortem evidence of rickets	In bones % H_2O	Ca wet	Ca dry	Histological diagnosis
601	♂	1	No milk	57	65	+	48·1	7·0	13·5	Osteoporosis + slight rickets
655	♀	2	„	53	57	–	48·7	6·7	13·2	„ „
654	♂	3	„	74	71	? +	48·7	6·7	13·2	Marked osteoporosis
						Av. no milk:	**48·5**	**6·8**	**13·3**	
			cc.							
602	♀	1	1	72	85	+	46·0	6·6	12·4	Osteoporosis + slight rickets
610	♀	1	1	57	70	+	45·5	8·0	14·8	„ „
664	♀	2	1	50	68	–	43·4	7·9	14·1	Osteoporosis
						Av. 1 cc.:	**44·9**	**7·7**	**13·7**	
603	♀	1	3	80	105	–	54·3	7·0	15·4	Osteoporosis + slight rickets
611	♂	1	3	72	108	+	43·3	8·4	14·9	„ „
663	♂	3	3	57	85	–	42·8	8·8	15·4	„ „
659	♂	2	3	62	81	–	43·8	7·7	13·7	Osteoporosis
						Av. 3 cc.:	**46·0**	**7·9**	**14·8**	
653	♂	2	5	68	122	–	45·8	8·9	16·5	Osteoporosis + slight rickets
605	♀	1	5	70	104	–	45·3	8·4	15·4	Slight osteoporosis
658	♂	3	5	55	102	–	44·2	9·4	17·0	„
608	♂	1	5	82	132	–	47·0	7·9	15·0	Osteoporosis + slight rickets
						Av. 5 cc.:	**45·3**	**8·6**	**15·9**	
606	♀	1	10	53	113	–	44·8	9·0	16·3	„ „
612	♂	1	10	81	145	–	45·4	8·0	15·1	„ „
661	♂	2	10	64	137	–	42·4	9·1	15·9	Osteoporosis
660	♂	2	10	60	115	–	44·5	9·8	17·7	„
						Av. 10 cc.:	**44·3**	**8·9**	**16·2**	
607	♀	1	15	76	124	–	42·5	9·7	17·0	„
657	♀	3	15	56	113	–	43·5	10·4	18·5	Nearly normal
						Av. 15 cc.:	**43·0**	**10·0**	**17·7**	
609	♀	1	20	72	133	–	41·1	11·4	19·4	„
656	♂	3	20	58	138	–	42·3	10·4	17·9	Osteoporosis
						Av. 20 cc.:	**41·7**	**10·7**	**18·6**	

Slightly improved % calcification with low doses as compared with Protocol B. High incidence of rickets.

Protocol D. Period IV. Sample XIII. Pasture.

Rat	Sex	Family	Dose	Initial weight	Final weight	Post-mortem evidence of rickets	In bones % H_2O	Ca wet	Ca dry	Histological diagnosis
744	♂	1	No milk	82	106	–	39·7	10·6	17·5	Osteoporosis
737	♀	1	„	66	87	–	40·2	9·6	16 1	Slight osteoporosis
747	♀	2	„	52	73	? +	47·3	8·1	15·5	Osteoporosis
						Av. no milk:	**42·4**	**9·4**	**16·3**	
			cc.							
739	♂	1	1	88	130	–	43·1	10·4	18·3	Normal
743	♀	2	1	76	90	–	35·7	12·3	19·1	Osteoporosis
						Av. 1 cc.:	**39·4**	**11·3**	**18·7**	
738	♀	1	2	60	103	–	38·9	11·1	18·2	Slight osteoporosis
748	♀	2	2	69	115	–	43·6	11·3	20·1	Normal
						Av. 2 cc.:	**41·5**	**11·2**	**19·1**	
740	♀	1	3	70	127	–	39·9	11·9	19·9	„
749	♀	2	3	53	107	–	48·5	9·5	18·6	„
						Av. 3 cc.:	**44·2**	**10·7**	**19·2**	
741	♀	1	5	66	103	–	Estimation lost			„
750	♀	2	5	60	112	–	43·9	11·5	20·5	„
754	♀	2	5	60	111	–	45·0	11·2	20·3	„
						Av. 5 cc.:	**44·4**	**11·3**	**20·4**	
742	♀	1	10	73	116	–	40·1	12·3	20·6	„
746	♀	2	10	57	97	–	45·0	11·6	21·0	„
						Av. 10 cc.:	**42·5**	**11·9**	**20·8**	
752	♀	2	15	67	132	–	41·8	12·4	21·2	„
753	♀	2	15	63	120	–	41·7	12·2	21·0	„
						Av. 15 cc.:	**41·7**	**12·3**	**21·1**	
745	♀	1	20	71	115	–	37·0	13·0	20·6	„
751	♂	2	20	75	167	–	43·5	12·1	21·4	„
						Av. 20 cc.:	**40·2**	**12·5**	**21·0**	

No rickets in control of milk-fed animals. High calcification with 5 cc. doses and over.

Protocol D 1. *Period IV. Sample XIV. Pasture.*

Rat	Sex	Family	Dose	Initial weight	Final weight	Post-mortem evidence of rickets	In bones % H_2O	Ca wet	Ca dry	Histological diagnosis
767	♂♀	1	No dose	63	62	+ +	50·0	7·0	14·6	Pronounced rickets
759	♀	2	„	78	60	16 ribs spontaneous fractures slightly enlarged, cch. junctions	58·1	6·6	15·7	Kept for demonstration
774	♀	3	„	58	77	+	42·6	7·6	13·4	Slight rickets
						Av. no milk:	**50·2**	**7·0**	**14·6**	
			cc.							
760	♀	1	1	64	80	? +	44·1	8·7	15·6	Osteoporosis
765	♀♀	1	1	74	83	–	43·3	7·8	13·9	„
763	♀♀	2	1	70	107	No fractures, cch. junctions normal	46·0	9·0	16·7	Kept for demonstration
770	♀	3	1	68	86	–	46·1	9·2	17·2	Slight osteoporosis
						Av. 1 cc.:	**44·9**	**8·7**	**15·8**	
761	♂♀	1	2	80	117	–	43·6	9·0	16·1	Normal
766	♀♀	3	2	78	109	–	42·5	8·2	14·3	„
757	♀	2	2	73	113	No fractures, cch. junctions normal	43·6	10·5	18·7	Kept for demonstration
758	♀	2	2	64	103	–	44·2	9·5	17·1	Normal
						Av. 2 cc.:	**43·5**	**9·3**	**16·5**	
771	♀	3	3	83	121	–	47·0	8·5	16·1	Osteoporosis
769	♀	1	3	78	127	–	47·5	9·3	17·7	Slight osteoporosis — nearly normal
764	♀	1	3	89	144	–	46·3	9·4	17·6	„ „
755	♀	2	3	92	136	–	44·7	10·2	18·5	Normal
						Av. 3 cc.:	**46·4**	**9·3**	**17·5**	
756	♀	1	5	74	136	–	44·6	10·5	19·0	„
780	♂	3	5	64	115	–	40·8	10·8	18·3	„
778	♂♀	3	5	78	145	–	44·7	9·2	16·7	„
779	♀	3	5	67	113	–	40·9	10·9	18·4	„
						Av. 5 cc.:	**42·7**	**10·3**	**18·1**	

No rickets in milk-fed animals. Improved calcification with low doses.

Protocol E. Period V. Sample XV. Dry fodder in the light.

Rat	Sex	Family	Dose	Initial weight	Final weight	Post-mortem evidence of rickets	In bones % H_2O	Ca wet	Ca dry	Histological diagnosis
840	♂	1	No milk	92	106	–	48·4	8·0	15·6	Osteoporosis + slight rickets
863	♂♀	2	„	79	108	–	48·3	7·9	15·3	„ „
826	♂	3	„	55	—	–	Died and eaten			Osteoporosis
855	♀	4	„	72	87	–	46·2	9·1	16·9	„
						Av. no milk:	**44·3**	**8·3**	**15·9**	
			cc.							
820	♂	3	1	76	106	+	50·1	8·0	16·1	Osteoporosis + slight rickets
835	♂♀	1	1	72	69	–	44·2	7·6	13·8	Osteoporosis
864	♀	2	1	72	81	–	44·3	8·7	15·6	„
						Av. 1 cc.:	**46·8**	**8·1**	**15·2**	
822	♀	3	2	82	119	–	49·8	8·7	17·5	Osteoporosis + slight rickets
839	♂	1	2	64	88	–	45·4	8·0	14·7	„ „
						Av. 2 cc.:	**47·6**	**8·3**	**16·1**	
825	♀	3	3	50	77	–	50·2	7·4	14·9	Osteoporosis
828	♀	3	3	79	88	–	43·4	9·7	17·2	„
866	♂	2	3	78	100	–	43·3	9·7	17·2	„
						Av. 3 cc.:	**45·6**	**8·9**	**16·4**	
832	♂	3	5	69	103	–	46·3	8·3	15·6	Slight osteoporosis
845	♂	1	5	73	124	–	46·5	9·8	18·4	Normal
861	♂♀	2	5	65	105	–	44·9	9·3	17·0	Slight osteoporosis
867	♂	4	5	76	117	–	44·6	9·8	17·8	„
						Av. 5 cc.:	**45·5**	**9·3**	**17·2**	
853	♀	4	8	63	107	–	45·1	10·2	18·5	Normal
851	♀	4	8	69	110	–	42·7	11·7	20·5	„
868	♂	2	8	62	126	–	46·8	9·0	16·9	„
						Av. 8 cc.	**44·8**	**10·3**	**18·6**	
858	♀	2	10	69	124	–	42·7	10·8	18·9	„
869	♀	2	10	56	111	–	47·0	9·6	18·1	„
870	♂	2	10	76	147	–	47·8	8·6	16·5	Slight osteoporosis
						Av. 10 cc.:	**45·8**	**9·6**	**17·8**	

Low % calcification with low doses as compared with pasture milk (Protocol D).

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XCV. THE POSSIBLE SIGNIFICANCE OF HEXOSE-PHOSPHORIC ESTERS IN OSSIFICATION.

PART II. THE PHOSPHORIC ESTERASE OF OSSIFYING CARTILAGE.

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In a previous communication by one of the present authors [Robison, 1923] it was shown that the bones and ossifying cartilage of young animals contain an enzyme which rapidly hydrolyses hexosemonophosphoric[1] ester and glycerophosphoric ester with liberation of free phosphate ions. The kidney was found to possess a similar hydrolytic power, but liver, spleen and pancreas were much less active in this respect, while muscle and the non-ossifying cartilage were almost or entirely without action on these monophosphoric esters.

A hexosediphosphatase, which hydrolyses at least one of the phosphoric acid groups of hexosediphosphoric ester, appeared to be more commonly distributed, being found in each of the tissues named above.

The suggestion was put forward that the former of these enzymes, the "monophosphoric esterase" present in bone, is concerned in the process of ossification by effecting the hydrolysis of phosphoric esters contained in the blood, thus bringing about a local increase in the concentration of inorganic phosphate ions, so that the product of the concentrations of phosphate and calcium ions becomes greater than the solubility product of calcium phosphate, which is thereupon deposited in the solid state.

The present paper is an account of further work on this enzyme.

Method of Extraction.

The tissues were thoroughly ground in a mortar with ten or twenty times their weight of water (or a 0·4 % solution of sodium bicarbonate) saturated with chloroform. In some of the later experiments these extracts (termed "10 %" and "5 %" respectively) were allowed to remain at 0° during 24 hours and were then filtered.

[1] Here and elsewhere in this paper "hexosemonophosphoric ester" refers to the compound isolated from the products of the fermentation of sugar by yeast juice [Robison, 1922] and not to the compound obtained by partial hydrolysis of the diphosphoric ester [Neuberg, 1918].

Such extracts prepared from bones have been kept at 0° during several months without suffering any serious diminution in their hydrolytic power, nor does there appear to be any rapid inactivation at room temperature. It is of course necessary that bacterial growth be inhibited by the addition of sufficient chloroform or other antiseptic.

Distribution of the enzyme.

In addition to the facts already reported some further information has been gained as to the occurrence of the enzyme in various animal tissues.

Bones. Active extracts have been obtained from the bones of all animals so far examined. These are: rat, rabbit, guinea-pig, kitten, chicken.

Teeth. The close similarity in the chemical composition of the inorganic portions of teeth and bones renders it very probable that the mechanism for the deposition of this material is similar in both cases. It was therefore interesting and significant to find that the phosphoric esterase is also present in teeth. Extracts prepared from the teeth of young and adult rabbits, rats and guinea-pigs usually contained the enzyme in even higher degree than those prepared from the bones of the same animals.

In one experiment "10 %" filtered extracts were prepared from the teeth and bones of young rats (three weeks old). 2 cc. of these extracts were added to 20 cc. of an $M/1000$ solution of sodium glycerophosphate and kept at 37°, the p_H being adjusted to 9·0–9·3. Colorimetric estimations of inorganic phosphate (*v.* p. 744) showed that at the end of $2\frac{1}{2}$ hours over 90 % of the glycerophosphate had been hydrolysed by both the teeth and bone extracts.

Intestines. The small intestines of rabbits, rats and guinea-pigs, washed with saline and then ground with water in the usual way, yielded extracts which hydrolysed phosphoric esters very rapidly, the activity being of the same order as that of kidney extracts. It is clear, therefore, that if phosphoric esters of the type under consideration are required by the organism they are not likely to be absorbed as such from the alimentary canal but must be formed within the body.

Variations in the activity of the tissues with the age of the animal.

Experiments were carried out with rats and rabbits of different ages, the hydrolytic power per g. of the different tissues being compared. The results were not very regular but showed in general that the superiority of the teeth and bone was most marked in very young, rapidly growing animals. In young adults the activity of the kidney was often equal to or greater than that of the bone, while in one very old rabbit the activity of the bone and teeth fell far below that of kidney and intestine and even of the liver. This falling off in the activity may be explained partly by the increased proportion of inorganic matter relative to the growing surfaces of the bone as well as by the reduction in the activity of the enzyme-secreting cells.

The optimum hydrogen ion concentration.

The exact determination by electrometric methods of the optimum hydrogen ion concentration has not yet been carried out, but sufficient information for our immediate purpose was furnished by the following experiment:

A number of tubes was prepared, each containing:
1 cc. { of a solution ($\overline{HP_1}$) of potassium hexosemonophosphate (containing 2·64 mg. P),
or a solution ($\overline{GP_1}$) of potassium glycerophosphate (containing 2·66 mg. P).
0·5 cc. of a 2½ % extract of epiphyseal cartilage of young rats (macerated but not filtered).
0·02 cc. chloroform.
0·1 cc. of a ·02 % solution of phenol red or thymol blue.
0·3 cc. $N/50$ NaOH or $N/50$ H_2SO_4 or H_2O as required to adjust the p_H.

The reaction was adjusted so that the tubes formed a series of varying p_H from 6·8–9·9. They were kept at 37° during 18 hours and the inorganic phosphate in each tube was then estimated by the modified Neumann method previously described [Robison, 1923]. Controls containing the solutions of phosphoric esters without cartilage extract and the latter without ester were treated in the same way. The very small amounts of inorganic phosphate found in these controls were subtracted from those found in the other tubes in order to determine the amount of hydrolysis effected by the enzyme.

Table I.

p_H before incubation	p_H after incubation	Inorganic phosphate (mg. P) less that in controls		% of phosphoric ester hydrolysed	
		$\overline{HP_1}$	$\overline{GP_1}$	$\overline{HP_1}$	$\overline{GP_1}$
Phenol red series:					
6·8	7·0	0·12	0·15	5	6
7·0	7·4	0·21	0·20	8	8
7·4	7·8	0·275	0·355	10	13
7·6	7·9	0·36	0·47	14	18
8·0	8·2	0·75	0·80	28	30
8·4	8·4	1·515	1·52	57	57
Thymol blue series:					
8·4	—	1·37	1·51	52	57
9·0	—	1·42	1·48	54	56
9·4	—	1·51	1·49	57	56
9·9*	—	0·82	0·21	31	8

* This value, being near the extreme limit of the colour range of thymol blue, was somewhat uncertain.

The activity of the enzyme is thus greatest in markedly alkaline solutions, the optimum p_H being 8·4–9·4 (determined colorimetrically). Between these limits the activity is practically constant, but on either side of them it falls off rapidly, the rate of hydrolysis at the normal p_H of the blood being only about one-fifth of that at the optimum.

At the same hydrogen ion concentration the rates of hydrolysis of hexosemonophosphoric ester and glycerophosphoric ester are the same.

In the experiments previously described the solutions of the esters were made just neutral to phenolphthalein before the addition of the tissue extracts, so that the final p_H was a little below the optimum in the region where a slight change in reaction makes considerable difference in the rate of hydrolysis.

We have therefore repeated some of these comparative tests at a p_H lying well within the limits for optimum activity of the enzyme. The results show the same marked superiority in the hydrolytic power of the bone extracts over those of the other tissues with the exception of the kidney and intestine. The difference between bone on the one hand and liver and pancreas on the other is even more pronounced at the higher p_H, which points to the probability that different enzymes are concerned.

In one experiment the tissues of two rats, 27 days old, were separately macerated with twenty times their weight of a 0·4 % solution of sodium bicarbonate, saturated with chloroform. These "5 %" extracts were used without filtration. A portion of the bone extract was diluted with an equal volume of sodium bicarbonate solution, thus making a "$2\frac{1}{2}$ %" extract.

Tubes were prepared containing

1 cc. { water, or solution ($\overline{HP}_1$) of potassium hexosemonophosphate (containing 2·64 mg. P), or solution ($\overline{GP}_1$) of potassium glycerophosphate (containing 2·66 mg. P).
0·5 cc. tissue extract.
0·1 cc. 0·4 % solution of thymol blue.
0·02 cc. chloroform.
0·1 cc. $N/10$ NaOH solution or water.

The p_H was adjusted to 9·0–9·2 and the tubes were kept during 18 hours at 37°, after which the inorganic phosphate was estimated by the modified Neumann method. The results are shown in Table II.

Table II.

Tissue	Extract %	Inorganic PO_4 (mg. P) after 18 hrs. at 37° less that in controls			% of phosphoric ester hydrolysed	
		H_2O	$\overline{HP}_1$	$\overline{GP}_1$	$\overline{HP}_1$	$\overline{GP}_1$
Bone cartilage (epiphyses)	5	·03	2·44	—	92	—
„ „	$2\frac{1}{2}$	·02	2·06	2·25	78	85
Rib cartilage (unossified)	5	·06	0·04	—	2	—
Kidney	„	·09	1·87	2·20	71	83
Liver	„	·03	0·27	0·22	10	8
Spleen	„	·03	0·46	0·51	17	20
Pancreas	„	·06	0·03	0·08	1	3
Muscle	„	·09	—	0·25	—	9

Proportionality between amount of enzyme and change effected does not of course exist when the latter amounts to 70–80 % of the total substrate, but it is clear that in the above experiment the activity of the epiphyseal cartilage relative to that of the kidney was about 2 : 1 and to that of the spleen, liver and pancreas decidedly greater than 8 : 1, 16 : 1 and 80 : 1 respectively.

The question whether the hydrolytic power of the different tissues with respect to the monophosphoric esters is due in each case to the same enzyme was further investigated by comparing the relative activities at different H^+ concentrations. The tests were carried out with "5 %" filtered extracts prepared from tissues of several rats of the same litter (10 weeks old), sodium glycerophosphate being the substrate. Four series of tubes were prepared;

in one the p_H was adjusted to 7·3 (indicator, phenol red), in the second to 8·3 (indicator, phenolphthalein), and in the third to 9·3 (indicator, thymolphthalein). The fourth series contained water in place of glycerophosphate and served as controls. The extracts of bone, teeth and kidney were used in a dilution of 1 in 5 (*i.e.* "1 %" extract) for the series at p_H 8·3 and p_H 9·3. The amounts of ester hydrolysed were thus rendered more comparable, in no case exceeding 40 % of the total substrate.

The inorganic phosphate set free in 18 hours at 37° was, in this experiment, estimated colorimetrically by the Briggs modification of the Bell-Doisy method, proteins being first precipitated by trichloroacetic acid. The ratios of the amounts of hydrolysis effected by each tissue at p_H 9·3 and 8·3 to that effected at p_H 7·3, as shown in Table III, confirm the supposition that the activity of the liver and pancreas and probably also of the spleen is due to a different enzyme from that in the bone and kidney.

Table III.

Tissue	Ratio of amounts of ester hydrolysed at p_H 7·3	8·3	9·3
Bone	1 :	3·5 :	5·5
Teeth	1 :	3·7 :	5·8
Kidney	1 :	3·0 :	5·1
Liver	1 :	0·6 :	1·0
Spleen	1 :	2·0 :	3·2
Pancreas	1 :	0·2 (?) :	1·5

Action of the bone enzyme on olive oil and other esters.

The marked difference between the bone enzyme and the pancreatic lipase and esterase was again brought out in the comparison of their actions on olive oil and other esters. The experiment was carried out at the same time as the preceding one and with the same 5 % tissue extracts.

The following solutions were used for the substrates:

(*a*) Olive oil emulsion containing 0·2 % bile salt.

(*b*) Triacetin solution (1 % by volume).

(*c*) Ethyl butyrate solution (1 % by volume).

5 cc. of the solution were added to 5 cc. of the 5 % tissue extract (bone or pancreas) or to the same volume of extract which had previously been heated to 100°. 0·5 cc. phenolphthalein solution (·1 %) was added to each tube of the series containing olive oil and 0·5 cc. phenol red solution (·02 %) to each of the others. The p_H was adjusted to 8·4 in each case and the tubes were placed in a thermostat at 37°. At short intervals (five minutes at first) the contents of the tubes were titrated to p_H 8·4 with 0·1 *N* sodium hydroxide solution and the total amounts thus added during 40 minutes and $3\frac{1}{2}$ hours are shown in Table IV. At the end of $3\frac{1}{2}$ hours alcohol was added to the tubes containing olive oil emulsion and the titration of the free acid carried out in the usual way. The amounts of sodium glycerophosphate hydrolysed

in 18 hours by 1 cc. extract are also shown for comparison with the amounts of the esters hydrolysed in 3½ hours by 5 cc.

Table IV.

0·1 *N* sodium hydroxide.

		Bone extract	Bone extract heated to 100°	Difference = amount hydrolysed	Pancreas extract	Pancreas extract heated to 100°	Difference = amount hydrolysed
Olive oil	40 mins.	—	—	—	—	—	—
	3½ hrs.	0·26	0·32	−0·06	22·1	0·33	21·77
Triacetin	40 mins.	0·33	0·07	0·26	1·46	0·07	1·39
	3½ hrs.	1·12	0·46	0·66	2·98	0·46	2·52
Ethyl butyrate	40 mins.	0·20	—	0·20	1·58	—	1·58
	3½ hrs.	1·06	—	1·06	1·97	0·07	1·90
Sodium glycerophosphate (+1 cc. bone extract, 18 hrs. at 37°)		—	—	2·56 mg. P = 0·82 cc. 0·1 *M* soln.	—	—	0·029 mg. P = 0·009 cc. 0·1 *M* soln.

The rate of hydrolysis of ethyl butyrate by the pancreas had fallen to zero before the end of 3½ hours, while that of triacetin had become very slow. This was probably owing to the effect of the reverse reaction since the hydrolysis was by no means complete. The figures for 40 minutes give therefore a fairer basis for comparison.

The bone extract thus hydrolysed ethyl butyrate, triacetin and glycerophosphoric ester at about the same rate, but had no action on olive oil. The pancreas extract, which hydrolysed the oil very rapidly, was also six or seven times as active as the bone extract towards ethyl butyrate and triacetin. Towards the phosphoric ester, however, its activity was less than 1/80 of that possessed by the bone extract.

Action of the bone enzyme on lecithin.

The proof that glycerophosphoric ester and hexosemonophosphoric ester are hydrolysed with equal rapidity by the bone enzyme, together with the fact that lecithin, which is a derivative of glycerophosphoric ester, is known to be present in the blood, while the presence of hexosemonophosphoric ester is still unproven, made it desirable to examine the action of the enzyme on lecithin.

A quantity of this substance was prepared from egg yolk and was used in the crude condition without removal of the kephalin. It was made up into an emulsion of about *M*/100 concentration with a 0·1 % solution of bile salt. Three flasks were made up as follows:

LB. 25 cc. *M*/100 lecithin emulsion + 5 cc. 5 % bone extract.
LO. 25 cc. *M*/100 lecithin emulsion + 5 cc. 5 % bone extract heated to 100°.
GB. 25 cc. *M*/100 sodium glycerophosphate solution containing 0·1 % bile salt + 5 cc. 5 % bone extract.

One drop of phenolphthalein was added to each flask and the reaction was adjusted till a faint pink tint was visible (p_H 8·3–8·4). 5 cc. of the liquid were at once removed from each flask and run into 1 cc. of 15% trichloroacetic acid solution. After shaking and filtering, a measured quantity of the

protein-free filtrate was taken for the estimation of inorganic phosphate by Briggs' method. The flasks were placed in a thermostat at 37° and the inorganic phosphate again estimated at intervals. The results are shown in Table V. In another experiment a solution similar to "LB" given above, but with phenol red in place of phenolphthalein, was kept for six hours at 37° without any change in the p_H being observed. It would seem therefore that neither the fatty acids nor phosphoric acid are set free from lecithin by the bone enzyme.

Table V.

	Inorganic PO_4 (mg. P) in 5 cc.		
Time at 37° (hrs.)	LO	LB	GB
0	None	0·035	0·042
$2\frac{1}{2}$	"	—	0·252
$22\frac{1}{2}$	"	0·033	0·702
Amount hydrolysed	—	None	0·660
	Total P (mg. P) in 5 cc.		
	1·46	—	1·32

Deposition of calcium phosphate in bones of rachitic animals.

We have continued the experiments on the deposition of calcium phosphate in the bones of rachitic animals of which brief mention was made in the previous paper [Robison, 1923]. It was there shown that the bones of rachitic rats contained the enzyme in a very high degree, the extracts being relatively more active than those of normal animals. This suggested the possibility that the immediate cause of the failure to deposit calcium phosphate in these bones might be the lack of the necessary substrate, some phosphoric ester, in the blood. If this were so, these poorly calcified bones, if immersed under suitable conditions in a solution of calcium hexosemonophosphate or calcium glycerophosphate might, by hydrolysis of the ester, show a deposit of calcium phosphate in those portions where normally calcification should have taken place. A considerable measure of success was realised at the first attempt, but a large number of trials had to be made before the best conditions were discovered. The chief difficulty lay in the fact that diffusion of the substrate into the bone is a very slow process compared with the constant supply of material by the blood stream to those points where it is required. Probably for this cause we were not successful in our attempts to carry out the experiment with solutions containing approximately the same amounts of calcium and total phosphorus as are found in the blood. By using stronger solutions of the ester and immersing the bones for a sufficient length of time, a considerable deposit of calcium phosphate was obtained, but this was limited by the simultaneous diffusion of the enzyme causing a general deposit of calcium phosphate in the tube and throughout the bone. In all our later experiments the p_H of the solution has been adjusted to lie within the optimum range for the enzyme. *i.e.* 8·4–9·4. Whether there is in ossifying cartilage some mechanism for raising the p_H of the plasma bathing the cells is a question that cannot at present be answered, but *in vitro* the deposition

of calcium phosphate certainly proceeds more rapidly under such conditions. Calcium hexosemonophosphate and calcium glycerophosphate were used as substrates with equal success.

Method of experiment. The bones and ribs used in these experiments were taken from young rats that had been fed during three to four weeks on a diet deficient both in the fat-soluble *A* factor and in phosphorus (McCollum diet No. 3143). They were removed and immersed in the solution as rapidly as possible after the animals were killed. In some cases the bones were used intact, while in others they were cut in half longitudinally, one half being used as a control. After immersion in the solutions of calcium hexosemonophosphate or calcium glycerophosphate at 37° for the specified time, they were rinsed several times with saline, fixed in formalin, dehydrated and embedded in the usual way. It was of course necessary that the sections should be cut without preliminary decalcification. Staining was carried out by immersing the section for 30 minutes in a 1 % solution of silver nitrate during exposure to bright light (von Kossar's method).

We take it that the staining of the calcified portion of bone by this method is to be explained thus. The silver nitrate reacts with the calcium phosphate which is very slightly soluble in water forming the still less soluble yellow silver phosphate which is then reduced to metallic silver by the action of the light, accelerated by the organic matter of the bone. If any soluble phosphates, chlorides or other salts, which form insoluble silver compounds, were present, these also would yield a black stain, but such compounds should be entirely removed in the preliminary treatment. We were, however, anxious to make certain that the new deposit observed in our experiments was really derived from calcium phosphate set free by the action of the enzyme on the phosphoric ester, and not to the presence of calcium glycerophosphate itself or any other salt retained by the cartilage merely by adsorption or in some form of physical or chemical combination. We therefore carried out a series of controls by immersing the other halves of the same bones, or similar bones from the same animals, in solutions of sodium chloride, sodium phosphate and calcium chloride. The results uniformly obtained after satisfactory conditions had been determined leave no doubt that fresh calcium phosphate had been deposited by the action of the enzyme on the phosphoric ester in those bones treated with the solutions of calcium hexosemonophosphate and calcium glycerophosphate. The controls were in all cases negative. The difference in the two groups was indeed at once apparent to the naked eye when the sections were stained. On microscopic examination it was seen that in the controls the broad zone of hypertrophic cartilage, characteristic of rickets, remained entirely uncalcified. In the sections of the bones that had been immersed in the solutions of the phosphoric esters this hypertrophic zone was deeply stained, and under higher magnification this was seen to be due to the presence of minute granules thickly deposited throughout the matrix of the cells. The deposit was most dense in the zone of provisional calcification, only a few

discrete particles being seen among the cells of the proliferating cartilage, and none at all in the resting cartilage.

In these bones the periosteum was also deeply stained, which led us to carry out a similar experiment with periosteum stripped from the bone of a rachitic rat before immersion in the solutions. After treatment with calcium glycerophosphate the sections showed a deep brown staining extending from the inner surface through about one-third of the tissue, while in the control no staining at all occurred.

The photomicrographs shown in Plates IV and V illustrate the results of these experiments.

Fig. 1, Plate IV. Section of half a humerus from a severely rachitic rat, after being soaked in disodium phosphate solution at 37° for eight hours (p_H 8·6–8·8).
RC = Resting cartilage.
PC = Zone of proliferating cartilage.
HC = Zone of hypertrophic cartilage, which is entirely uncalcified.
M = Metaphisis.
Co = Cortex.
Stained with silver nitrate and counterstained with eosin.
Magnification × 15.

Fig. 2, Plate IV. Section of the other half of the same humerus as in Fig. 1, but soaked in a solution of calcium hexosemonophosphate at 37° for eight hours (p_H 8·6–8·8). The entire zones of proliferating and hypertrophic cartilage are here deeply stained. There is a dense precipitation of granules of calcium phosphate in the matrix of the cells. With a higher magnification granules can also be seen scattered lightly over the surface of the cells themselves. The general appearance seems to indicate that considerable diffusion of the enzyme has taken place during the lengthy immersion.
Stained with silver nitrate and counterstained with eosin.
Magnification × 15.

Fig. 3, Plate IV. Section of rib of slightly rachitic rat, immersed intact in a solution of disodium phosphate for 12 hours at 37° (p_H 8·4). There is no fresh calcium phosphate deposition in either the periosteum or the zone of hypertrophic cartilage.
Stained with silver nitrate; no counterstain.
Magnification × 30.
Sections of other control ribs from the same rat soaked in (1) sodium chloride, (2) calcium chloride present the same appearance.

Fig. 4, Plate IV. Rib from the same rat as in Fig. 3. Immersed intact in calcium glycerophosphate solution for 12 hours at 37° (p_H 8·4). The zone of hypertrophic cartilage and the periosteum are stained dark brown. Some deposit is also to be seen in the lower part of the zone of proliferating cartilage.
Stained with silver nitrate; no counterstain.
Magnification × 30.
Although the period of immersion was somewhat longer than with the divided bone shown in Figs. 1 and 2, Plate IV, diffusion would not seem to have occurred to the same extent in these intact bones.

Fig. 5, Plate V. A drawing made from the portion of Fig. 3 (Plate IV) enclosed in the rectangle.
Magnification × 320.
No granules are to be seen.

Fig. 6, Plate V. Drawing made from the part of the rib in Fig. 4 (Plate IV) enclosed in the rectangle, showing the relative size and density of the calcium phosphate granules deposited in the different zones of the cartilage.
Magnification × 320.

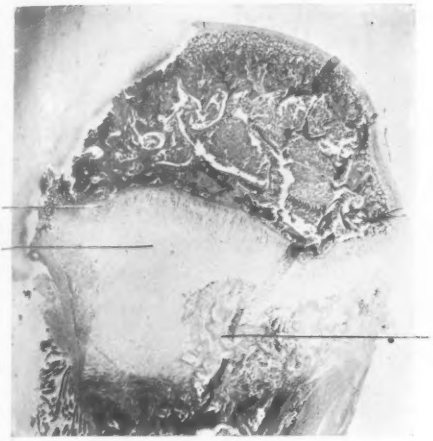

Fig. 1.

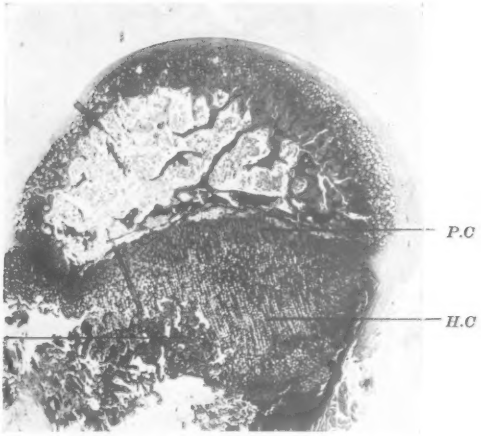

Fig. 2.

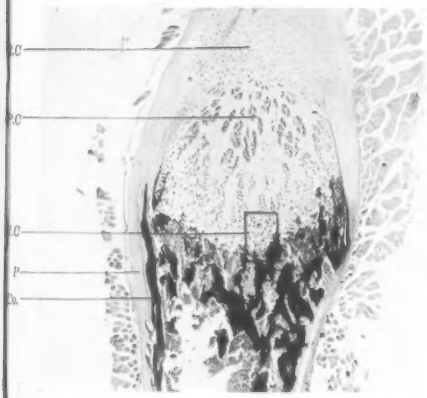

Fig. 3.

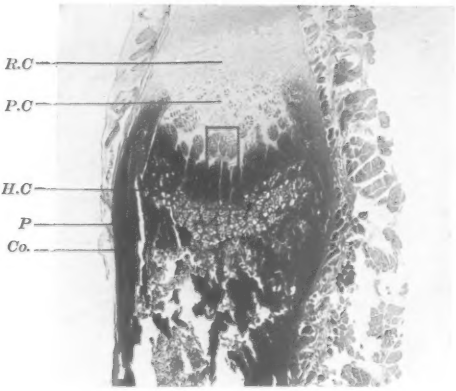

Fig. 4.

Fig. 5.

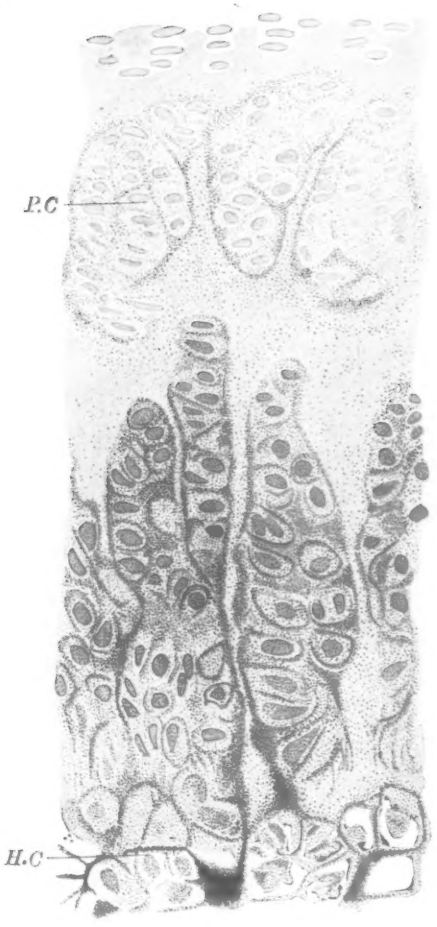

Fig. 6.

Discussion.

The simplest way of considering the relationship between the inorganic portion of bone and the fluid by which it is bathed, is as that of a solid in contact with its saturated solution. If for the moment we assume that the solid is tricalcium phosphate, the reactions taking place are

$$Ca^{++} + PO_4^{\equiv} \longleftrightarrow \underset{\substack{\text{dissolved}\\\text{un-ionised}}}{Ca_3(PO_4)_2} \longleftrightarrow \underset{\text{solid}}{Ca_3(PO_4)_2},$$

and the equation

$$[Ca^{++}] \times [PO_4^{\equiv}] = K\,[Ca_3(PO_4)_2] = k \text{ (solubility product)}$$

should hold for this system as for a saturated solution of the salt in pure water, except that the value of k will not necessarily be the same but may be modified by the other substances dissolved in the tissue fluid. If the concentrations of the Ca and PO_4 ions are such that the product is greater than k more solid calcium phosphate will be deposited, whereas if the product is less than k some of the solid will be re-dissolved. Both deposition and re-solution occur in the body, the former during normal bone formation, the latter under special conditions such as that of pregnancy if the increased demand of the organism for calcium is not satisfied by the diet. The equilibrium is conditioned by the concentration of the above ions and not directly by that of the total calcium and phosphate present. According to Rona and Takahashi [1913] and Clark [1923], a portion of the calcium of serum is present in non-diffusible form, probably in combination with protein. This still leaves the concentration of diffusible calcium in the serum greatly in excess of that corresponding with the solubility of $CaCO_3$, under similar conditions with regard to the HCO_3 content and p_H, as determined by Rona and Takahashi [1913], from which they concluded that serum forms a metastable supersaturated solution of calcium bicarbonate (? carbonate). Their solubility figure, 22 mg. Ca per litre at 18°, has been confirmed by Brinkman and van Dam [1920] who also devised a method by which the calcium ions alone could be estimated. Measured amounts of oxalate solution were added until the product of calcium and oxalate ions exceeded the solubility product of calcium oxalate, which was precipitated, giving rise to a turbidity. In this way they determined the ionised calcium in serum and found amounts corresponding closely with the solubility figure of 22 mg. per litre.

The whole of the inorganic phosphate in serum was found by Rona and Takahashi [1913] to be present in diffusible form, but of this phosphate only a very small proportion will be present as $[PO_4^{\equiv}]$ at the p_H of the blood.

To consider the plasma as supersaturated with respect to calcium carbonate and phosphate does not seem justified until more is known of the factors influencing the degree of ionisation and the solubility product. A supersaturated solution might, it is true, deposit calcium salts only on coming into contact with the solid phase, *i.e.* bone, but this would not explain the

beginning of ossification in embryonic cartilage, nor the ease with which the organism draws on its store of calcium salts in the skeleton when it has more urgent need of them for other purposes.

If then the plasma or tissue fluid be considered as a saturated solution of these salts, we must look for some factor by which a localised increase in the concentration of one or both ions may be brought about. With the interesting possibility of an increase in the proportion of ionised calcium we are not for the present concerned. Experiments on this point have been reported by Clark [1923].

The concentration of $PO_4^{\equiv}$ ions might be increased in two ways: 1) by an increase of total inorganic phosphate, (2) by a shift of p_H towards the alkaline side, thereby increasing the $PO_4^{\equiv}$ at the expense of HPO_4^{-} ions. As we have shown, the bone enzyme supplies one factor by which, in presence of a suitable phosphoric ester, the concentration of inorganic phosphate and therefore of $PO_4^{\equiv}$ ions could be increased. The experiments described in this paper confirm the previous suggestion [Robison, 1923] that this enzyme is secreted in an active condition in those parts of the bone, and only those parts, where ossification normally takes place.

The demonstration of calcium phosphate deposition, *in vitro*, in bones taken from rachitic animals and immersed in solutions of suitable phosphoric esters, not only strengthens the probability that the enzyme is actively concerned in ossification, but also furnishes information with regard to the areas of the bone in which it is secreted. The probability that some diffusion of the enzyme takes place after removal of the bone and during the experiment must, however, be reckoned with, and comparison of the stained sections obtained after immersion of the bones for different lengths of time leads to the conclusion that in those experiments which yielded the most deeply stained sections (Fig. 2, Plate IV) such diffusion had occurred.

To what extent, then, can the stained areas in sections obtained in the less prolonged experiments such as those shown in Figs. 4 and 6, Plates IV and V, be taken as indicating the normal location of the enzyme? By examining the apparently unstained sections obtained in the shortest experiments (the bones immersed for two hours only) under high magnification it was seen that a slight sprinkling of fine particles was present in the same areas in which a more obvious deposit was found, in bones from the same rat after eight hours' immersion.

Without wishing to make too definite an assertion, we think that this indicates the probability that the enzyme had actually been secreted throughout this area during life, and had not merely found its way by diffusion from some other part of the bone after its removal from the animal.

The fact that the enzyme is most active at a p_H between 8·4 and 9·4 suggests a further interesting possibility, that osteoblasts or hypertrophic cartilage cells have the power to raise the p_H of the tissue fluids. Such a change of reaction would at once increase the concentration of $PO_4^{\equiv}$ ions by shifting

the equilibrium between them and the $HPO_4^=$ and would at the same time increase the activity of the enzyme. It would also shift the equilibrium between $HCO_3^- \longleftrightarrow CO_3^=$ further to the right and thus cause a deposit of calcium carbonate which might conceivably account for the presence of this substance to the extent of about 10 % in the inorganic portion of bone.

We have attempted to obtain proof of such an alkaline reaction in the ossifying cartilage by intra-vital injections of thymol blue, phenol red and other indicators. Rats tolerated a series of such injections into the heart but though, on removal of the bones immediately after death, we found the surrounding muscles stained, we have not yet succeeded in obtaining penetration of the stain into the cartilage. Macerated extracts of bones, after standing at 37°, frequently had a p_H of about 8·4, but this might be due to re-solution of the calcium salts from the solid bone. These experiments are being continued.

As regards the chemical composition of the inorganic deposit in bone and teeth, it has been suggested by Gabriel [1894] and others that the chief constituent is a combined carbonate-phosphate of calcium. Some of Gabriel's analyses are shown below and illustrate the very close agreement in chemical composition of these deposits.

Percent. of	Human bone	Ox bone	Ox teeth
CaO	51·31	51·28	50·76
MgO	0·77	1·05	1·52
K_2O	0·32	0·18	0·20
Na_2O	1·04	1·09	1·16
Water of crystallisation	2·46	2·33	2·21
P_2O_5	36·65	37·46	38·88
CO_2	5·86	5·06	4·09
Cl	0·01	0·04	0·05
Water of constitution...	1·32	1·37	1·27
Total...	99·74	99·86	100·14

Bassett, Jr. [1917] has shown that the solid phase in equilibrium with solutions containing calcium and phosphate under the conditions found in normal serum is hydroxyapatite $(Ca_3P_2O_8)_3Ca(OH)_2$, a compound even more basic than tertiary calcium phosphate. He considers that the mineral constituents of bone consist in the main of this compound mixed with a certain amount of calcium carbonate. The traces of magnesium, sodium and potassium and of chloride he considers to be carried down by adsorption. The solubility relationships of the various calcium phosphates have not yet been satisfactorily determined, but it is somewhat surprising to find suggested in several recent papers the idea that the solubility product of $CaHPO_4$ is the limiting factor in the deposition of bone. This conception is developed at some length by Howland and Kramer [1923] who have attempted to show that rickets is associated with a low product of calcium and phosphate concentrations in the blood.

They recognise that calcium exists in the bone as tertiary calcium phosphate, but do not understand how this can be formed, and state that "While

the transformation from the secondary to the tertiary phosphate can be brought about with ease in the test-tube by lowering the carbon dioxide tension it does not appear likely that such a profound alteration could take place in the body (change of reaction to p_H 12) as is required to bring about a precipitation of tertiary calcium phosphate."

In this argument they forget that, although at p_H 7·4 the concentration of $PO_4^{\equiv}$ may be extremely small in relation to that of $HPO_4^{=}$, it is nevertheless not "insignificantly small," but is the determining factor as regards deposition of the solid phase owing to the very slight solubility of the tertiary salt.

In another paper by Kugelmass and Shohl [1924] there appears the statement: "At the hydrion concentration of blood $CO_3^{=}$ and $PO_4^{\equiv}$ are negligible and so the equilibria involve only the ions Ca^{++}, H^+, HCO_3^-, $HPO_4^{=}$ and $H_2PO_4^-$ and the molecules CO_2, H_2CO_3, $Ca(HCO_3)_2$, $Ca(HPO_4)$ and $Ca(H_2PO_4)_2$."[1]

The equilibria between un-ionised, dissolved $Ca_3(PO_4)_2$ and $CaHPO_4$ and the ions derived from them are expressed by the following equations:

$$(1)\quad [Ca^{++}] \times [PO_4^{\equiv}] = K_1 [Ca_3(PO_4)_2].$$

$$(2)\quad [Ca^{++}] \times [HPO_4^{=}] = K_2 [CaHPO_4].$$

$$(3)\quad [H^+] \times [PO_4^{\equiv}] = K_3 [HPO_4^{=}].$$

If k_1 and k_2 are the solubility products of $Ca_3(PO_4)_2$ and $CaHPO_4$ respectively, then if the concentrations of calcium and total inorganic phosphate are sufficiently increased, deposition of solid salt will occur (if supersaturation be excluded) when either

$$[Ca^{++}] \times [PO_4^{\equiv}] > k_1 \text{ the solid being in this case } Ca_3(PO_4)_2$$

or $$[Ca^{++}] \times [HPO_4^{=}] > k_2 \quad \text{,,} \quad \text{,,} \quad CaHPO_4.$$

Which of these conditions is realised depends on the values of k_1, k_2, K_3 and $[H^+]$.

The solution will come into equilibrium with solid $Ca_3(PO_4)_2$ or will deposit this salt if $\frac{[PO_4^{\equiv}]}{k_1} > \frac{[HPO_4^{=}]}{k_2}$, or, since $[PO_4^{\equiv}] = \frac{K_3 [HPO_4^{=}]}{[H^+]}$, if $\frac{K_3}{k_1 [H^+]} > \frac{1}{k_2}$, *i.e.* if $[H^+] < K_3 \frac{k_2}{k_1}$.

There will therefore be a limiting value for $[H^+]$ below which $Ca_3(PO_4)_2$ is deposited and above which $CaHPO_4$ is deposited. This value depends on the constants $k_1\ k_2\ K_3$ and need not correspond with a p_H of 12 as suggested by Howland and Kramer. That the limiting p_H is much lower than that of normal blood has long been known but was demonstrated very simply

[1] In the same paper these authors make use of Walker and Cormack's [1900] value for the first dissociation constant of H_2CO_3 ($3{\cdot}04 \times 10^{-7}$) and state that their own experiments confirm Walker and Cormack's conclusion that the fraction of total CO_2 existing in solution as H_2CO_3 is greater than 0·5. They ignore the work of Thiel and Strohecker [1914] who have shown that in a 0·00812 *M* solution of carbon dioxide only 0·67 % of the total CO_2 is present as carbonic acid, and that the dissociation constant of this acid is 5×10^{-4}, that is, it is twice as strong as formic acid.

by the following experiment. 10 cc. of a solution containing 10 mg. of phosphorus in the form of KH_2PO_4 and 30 mg. Ca in form of $CaCl_2$ was measured into a number of tubes, and varying amounts of 0·1 *N* NaOH solution added to each. Methyl red or phenol red was added to the tubes, which were then corked and kept at 37° during five days with frequent shaking. At the end of this time the p_H of the supernatant liquid in each tube was noted and was as follows:

cc. 0·1 *N* NaOH	p_H	
3·0	4·5	3·23 cc. *N*/10 NaOH required to form $R^1_2HPO_4$
6·3	5·0	
6·5	5·2	
6·8	5·4	6·46 cc. *N*/10 NaOH required to form $R^1_3PO_4$
7·0	5·8	
7·3	6·8	
7·6	7·8	

In absence of calcium the addition of 3·23 cc. 0·1 *N* NaOH would have raised the p_H to about 9·0 and to about the same value had a portion of the phosphate been precipitated as $CaHPO_4$. But double the above amount of NaOH was added without raising the p_H above 5·2 which proves that at this p_H the solid deposited must have been tertiary calcium phosphate or a still more basic compound. By the sudden addition of a large excess of calcium or phosphate, $CaHPO_4$ may of course be thrown down, but the solution will not be in true equilibrium with the solid phase, and some of the $CaHPO_4$ must re-dissolve and $Ca_3(PO_4)_2$ be precipitated until the p_H is thereby lowered below the limiting value (probably below 5·0).

For the sake of greater lucidity in the above paragraphs, we have considered HPO_4 and PO_4 ions alone, but the argument applies equally to other ions such as those derived from the still more basic hydroxyapatite, if, as Bassett suggests, this, and not the tertiary phosphate, is the compound present in bone.

Summary.

The phosphoric esterase discovered in bone and ossifying cartilage is also present in equally high degree in the teeth, especially those of very young animals.

The optimum p_H for this enzyme lies on a flat curve between 8·4 and 9·4, a reaction which is in other ways favourable for the precipitation of tertiary calcium phosphate and calcium carbonate.

The bone enzyme also hydrolyses ethyl butyrate and triacetin, but has no action on olive oil. The enzyme is however distinct from the esterase of pancreas (and probably also those of the liver and spleen) which is relatively inactive towards phosphoric esters.

Deposition of fresh calcium phosphate has been demonstrated in bones taken from rachitic rats and immersed in solutions of calcium hexosemonophosphate or calcium glycerophosphate at 37° and p_H 8·4–9·4.

These experiments further support the belief that the enzyme is actively concerned in ossification and also furnish evidence that the enzyme is secreted in the region of the osteoblasts and hypertrophic cartilage cells.

The high optimum p_H of the enzyme further suggests the possibility that the reaction of the tissue fluids in this region may by some mechanism be rendered more alkaline than that of the blood, thereby increasing the activity of the enzyme and providing favourable conditions for the deposition of calcium salts.

We have much pleasure in expressing our best thanks to Dr H. Goldblatt for his help in the histology of the earlier experiments.

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XCVI. THE POSSIBLE SIGNIFICANCE OF HEXOSE-PHOSPHORIC ESTERS IN OSSIFICATION. PART III.

THE ACTION OF THE BONE ENZYME ON THE ORGANIC PHOSPHORUS COMPOUNDS IN BLOOD.

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The occurrence in bone and ossifying cartilage of an enzyme which hydrolyses hexosephosphoric esters is not sufficient evidence that such esters are concerned in the process of ossification, for it must further be shown that they are present in the blood and are available wherever calcium phosphate is being deposited. The belief that this would prove to be the case was based on the fact that considerable quantities of acid-soluble organic phosphorus compounds are known to be present in blood, chiefly in the corpuscles, though small amounts have also been found in the plasma. The following averages are taken from Bloor's [1918] analyses of human blood (males).

mg. P per 100 cc.

			Acid-soluble	
	Total	Lipin	Inorganic	Other forms
Corpuscles	79	18·0	5·9	55
Plasma	10·2	7·0	2·7	0·5

The chemical composition of these acid-soluble compounds is still unknown, but their hydrolysis on boiling with dilute acids indicates that they are esters of phosphoric acid. The evidence produced by Embden and his collaborators that the lactacidogen of muscle is a hexosediphosphoric ester suggested that this or similar compounds might occur in blood. The diphosphoric ester itself could not serve as the required substrate, as it is hydrolysed by an enzyme present in almost all tissues including muscle and non-ossifying cartilage, but both mono- and di-phosphoric esters may be formed in the metabolism of carbohydrate within the animal body, as they are formed during fermentation of sugar by yeast juice. Thus a hexosemonophosphoric ester, synthesised perhaps in the liver, may not only play a part in ossification but, being also absorbed by the muscle, may be there converted into the lactacidogen or alternatively into glycogen. These speculations carry us, however, beyond our present knowledge and it must be granted that glycerophosphoric ester would serve as well as hexosemonophosphoric ester in the suggested scheme of ossification, since these two compounds are hydrolysed with equal rapidity by the bone enzyme [Robison and Soames, 1924].

The complete answer to this problem requires the isolation and identification of the various phosphorus compounds occurring in the blood and considerable progress has been made in this direction, but the experiments described in this paper prove definitely that the blood contains phosphoric esters which are rapidly hydrolysed when brought into contact with the bone enzyme under suitable conditions.

The presence of the necessary substrate is thus demonstrated and the probability that the enzyme plays a part in the ossification process is thereby increased.

The positive evidence so far obtained refers almost entirely to the corpuscles and the objection may be raised that calcium deposition must be determined ultimately by the concentration of calcium and phosphate ions in the plasma, in which the amount of phosphoric ester must in any case be very small. If the concentrations of the ester in plasma and corpuscles are in equilibrium the corpuscles would act as reservoirs. Moreover a very small amount of ester in plasma saturated with calcium phosphate would be sufficient to bring about deposition of the solid phase by increasing the concentration of phosphate ions when hydrolysis occurred. Some experiments here described furnish evidence of the correctness of this view but the difficulty remains for the present a real one.

The combined phosphoric acid which is readily hydrolysed by the bone enzyme represents 14 % to 36 % of the total organic phosphorus in the acid-soluble fraction. The remainder, though very resistant to the action of the enzyme, is hydrolysed by boiling with dilute acids. At least two different acid-soluble phosphoric esters are therefore present and a method by which the respective amounts of each may be estimated with a fair degree of accuracy has been worked out. This method is being applied to the investigation of phosphorus compounds in the blood in certain conditions of health and disease in the hope that the further information thus obtained may throw some light on the problems of phosphorus and of carbohydrate metabolism.

The phospholipins, although derivatives of glycerophosphoric ester, are not acted on by the bone enzyme.

Action of the bone enzyme on the acid-soluble phosphorus compounds of blood.

The first experiments were carried out with sheep's blood (100–200 cc.), corpuscles and plasma being separately examined. Proteins were precipitated with trichloroacetic acid and the filtrates were repeatedly extracted with ether to remove the excess of this acid and also any phosphorus compounds of lipin nature. The neutralised protein-free filtrate from the laked corpuscles was treated with bone extract and kept at 37° for 24 hours. At the end of this period a decided increase was observed in the amount of inorganic phos-

phate (estimated by the modified Neumann method), showing that hydrolysis had occurred. Similar treatment of the plasma filtrate yielded inconclusive results.

The extraction of the filtrate with ether was very troublesome and was soon given up as it was found that sodium trichloroacetate did not interfere with the activity of the enzyme and that the ethereal extracts never contained more than a trace of phosphorus.

In all our later experiments the inorganic phosphate was estimated colorimetrically by the Briggs modification of the Bell-Doisy method, thus enabling us to use much smaller quantities of blood. The method of experiment was modified from time to time as experience suggested but only the procedure finally adopted will be described in detail.

Method of experiment. For the investigation of whole blood 10–20 g. was the amount usually taken, although satisfactory experiments have been carried out with as little as 5 g. Mixed venous and arterial blood was obtained by severing the carotid and jugular after anaesthetising the animal[1]. In other cases the blood was taken from a vein or from the carotid with the aid of a canula. The blood was received in a weighed vessel containing 1–2 cc. of a 1 % solution of potassium oxalate. It was immediately weighed and was then transferred to a stoppered cylinder and laked by adding three to four volumes of distilled water. Proteins were precipitated by the addition of a 25 % solution of trichloroacetic acid in amount equal to the original volume of the blood. Water was added to make the total volume equal to seven times the weight of the blood, frothing being reduced by the aid of the minimum quantity of capryl alcohol. The liquid was vigorously shaken and allowed to stand for ten minutes, after which it was filtered through a No. 30 Whatman paper into a second dry stoppered cylinder. The volume (v) was noted and the filtrate then neutralised by the cautious addition of a 40 % solution of sodium hydroxide, phenolphthalein being used as indicator. By the addition of water the new volume was made equal to 7·5/7 v. Up to this point the operations were carried out with the utmost expedition owing to the possibility of hydrolysis of phosphorus compounds and consequent increase in the inorganic phosphate. In the neutralised filtrate no appreciable hydrolysis occurs at room temperature while even at 37° such hydrolysis, if it takes place at all, is extremely slow.

The bone extract was prepared by thorough maceration of the bones of young rats or other animals with twenty times their weight of water saturated with chloroform. After standing overnight in the cold room these extracts were filtered and were then kept at 0° in presence of sufficient chloroform. The same extract served for a series of experiments extending over a month or longer and showed no loss of activity.

[1] It is possible that the effects of anaesthesia and the method by which the blood was subsequently laked may have given rise to changes in the amounts of phosphorus compounds. *v.* Martland and Robison [1924].

For the enzyme experiments flasks were prepared containing the neutralised blood filtrate, bone extract, etc. in the following proportions. (The actual quantities varied, but were usually less than those shown.)

	FB Filtrate + enzyme cc.	FO (control 1) Filtrate + water cc.	OB (control 2) Water + enzyme cc.
Blood filtrate (dilution 1 g. blood in 7·5 cc.)	100	100	—
5 % bone extract	10	—	10
Water	—	10	100
Chloroform	1	1	1
Additions for adjustment of p_H (indicator, alkali, etc.) ...	1	1	1

A fourth flask containing filtrate + boiled enzyme was added to the above when the quantity of blood was sufficient, and the activity of the enzyme was tested in a fifth flask (MB) in which the blood filtrate was replaced by the same volume of an $M/1000$ solution of sodium hexosemonophosphate or sodium glycerophosphate. For the adjustment of the p_H either phenolphthalein or thymolphthalein was used. These indicators being colourless in acid solutions did not interfere with the later colorimetric estimations. By using phenolphthalein and adding alkali until a faint pink colour was visible, a p_H near the lower limit of the optimum range was obtained, but unless the reaction was readjusted frequently, the rate of hydrolysis was apt to be somewhat irregular. With thymolphthalein a p_H of 9·0–9·3 was obtained. This was well within the optimum range but carried the disadvantage of a somewhat greater tendency towards hydrolysis in the controls. The flasks were placed in a thermostat at 37·5° and at intervals the inorganic phosphate was estimated in measured volumes of their contents. For the first estimation portions were removed immediately after mixing the contents of the flasks and before the adjustment of the p_H, the necessary volume correction (1 %) being applied to the amount of phosphate found. 5 cc. of the liquid were pipetted into a test-tube containing 1 cc. of N sulphuric acid[1] to precipitate the proteins of the bone extract. (For "OB" 1 cc. of a 15 % solution of trichloroacetic acid was used.) The tubes were shaken and allowed to stand during ten minutes, after which the contents were filtered and 5 cc. of the final filtrates taken for phosphate estimation. This quantity corresponded with $\frac{5}{6} \times 5 \times \frac{100}{111} \times \frac{1}{7{\cdot}5} = 0{\cdot}50$ g. blood.

The total acid-soluble phosphorus was also estimated in the final filtrates by ignition with nitric and sulphuric acid. Hydrolysis was usually complete in about six hours but the experiment was continued for several hours longer, the p_H being readjusted when necessary. If the duration was extended to 24 hours the results were apt to be complicated by slight irregular increases in the inorganic phosphate, due partly to chemical hydrolysis, but possibly

[1] Sufficient sodium trichloroacetate was already present in the blood filtrates. The addition of further trichloroacetic acid at this stage leads to errors in the colorimetric measurements [Martland and Robison, 1924].

also to the very slow enzymic hydrolysis of the second ester. The results of a typical experiment are given in detail below.

No. 33. Whole blood of an adult rabbit taken from carotid and jugular. Hydrolysis by 5 % bone extract at 37·5°. p_H 9·3.

Inorganic phosphate (mg. P) in 5 cc. final filtrate—equivalent to 0·5 g. blood.

	Time in hours							Increase after hydrolysis	Total P, mg.
	0	½	1	2	3	5	10		
FB. Blood filtrate + bone extract	·0475	·055	·062	·0685	·074	·080	·080	·0325	—
FO. Blood filtrate + water	·024	—	—	—	—	·025	·024	0	·155
OB. Water + bone extract	·023	—	—	—	—	·023	—	0	·023
MB. *M*/1000 sodium hexosemonophosphate + bone extract	·025	·053	·071	·092	·103	·115	·130	·105	·133

	mg. P per 100 g. blood	
Total acid-soluble phosphorus	= 31·0	
Inorganic phosphate	= 4·8	
Phosphoric esters	= 26·2	
of which (*a*) esters hydrolysed by bone enzyme	= 6·5	= 24·8 % of organic P
(*b*) esters not hydrolysed by bone enzyme	= 19·7	= 75·2 % ,,

→ Time in hours for curve C

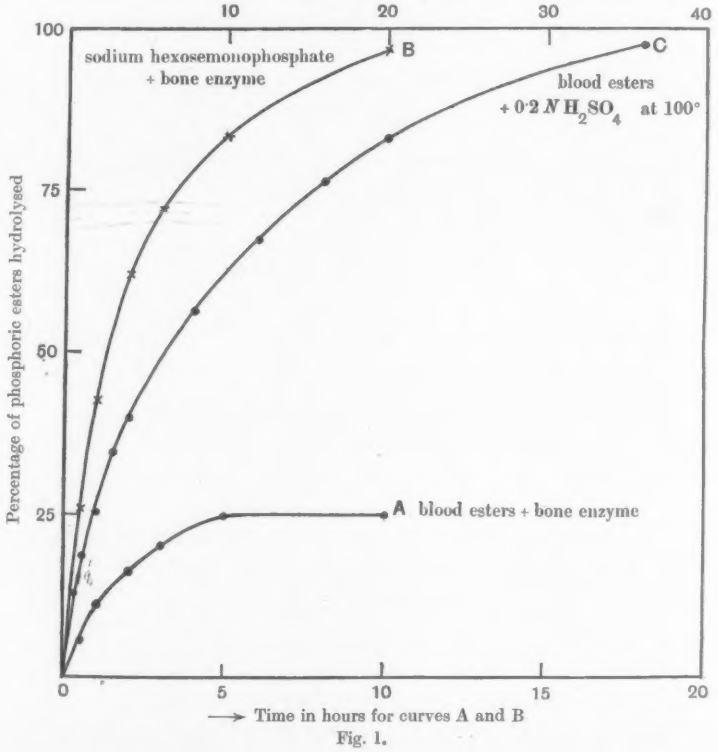

Fig. 1.

The results are shown graphically in Fig. 1 in which curve A expresses the amount of hydrolysis of the blood esters calculated as a percentage of the total organic acid-soluble phosphorus, and curve B the amount of hydrolysis of hexosemonophosphoric ester also as a percentage of the total ester. Curve C shows the hydrolysis of the blood esters by dilute acids and will be referred to later.

Table I. *Distribution of phosphorus in the acid-soluble compounds of blood.*

				mg. P per 100 g. blood					
No. of exp.	Animal	Age	Type of blood	Total acid soluble	Inorganic	Organic	Hydrolysed by bone enzyme	Not hydrolysed by bone enzyme	% of organic P hydrolysed by bone enzyme
9	Cockerel	6 months	Mixed	30·1	7·1	23·0	3·2	19·8	14
10	Rats	24 days	,,	38·5	10·9	27·6	9·5	18·1	34
8	,,	3 months	,,	28·7	4·5	24·2	7·4	16·8	31
11	,,	3 ,,	,,	27·8	5·0	22·8	7·4	15·4	32
12	,,	2½ ,,	,,	26·0	7·4	18·6	4·7	13·9	25
	,,	9 ,,	,,	29·1	5·4	23·7	5·4	18·3	23
15	,,	3 ,,	,,	33·6	5·4	28·2	6·0	22·2	21
	,,	9 ,,	,,	38·5	6·8	31·7	4·9	26·8	15
6	Rabbit	Adult	,,	28·4	5·4	23·0	7·0	16·0	30
17	,,	3½ months	,,	44·1	8·5	35·6	9·9	25·7	28
	,,	18 ,,	,,	37·1	7·8	29·3	6·5	22·8	22
33	,,	Adult	,,	31·0	4·8	26·2	6·5	19·7	25
42–49	,,	Young adults	Average of 7	38·4	6·2	32·2	10·5	21·7	32
27	,,	Adult	Venous (ear)	32·0	5·1	26·9	8·5	18·4	32
			Arterial (carotid)	32·3	7·8	24·5	8·7	15·8	35
16	Child (O. H.) ♀	6 years	Venous (arm)	21·9	4·3	17·6	6·4	11·2	36
28	Child ♀	10½ ,,	,,	22·8	4·1	18·7	5·7	13·0	30
	Child ♂	11 ,,							
26	Man (R. R.)	Adult	,,	25·2	2·6	22·6	6·0	16·6	27
16	,,	,,	,,	26·6	3·3	23·3	7·0	16·3	30
	Man (S. S. Z.)	,,	,,	21·9	3·2	18·7	6·1	12·6	33

Similar experiments have been carried out with the blood of many animals of different species and age and the main results are summarised in Table I. Except when otherwise stated mixed venous and arterial blood from one or more animals was used. Of the human subjects both the adults were normal, the child O.H. was suffering from slight vaginitis and the other children were congenital syphilitics but were not suffering from active disease at the time. Except in the case of O.H. the blood was taken between 10 a.m. and 11 a.m. about two hours after breakfast. The figures for human blood show less variation than the rest but this may be due to the fact that it was in every case taken from the arm vein and possibly greater differences would be found in blood before passage through the capillaries. Experiment 27 was planned to obtain information on this point although the ear vein was probably not the most suitable one for this purpose owing to the relatively low metabolic activity of the tissue through which the blood had passed. Blood was first taken from the ear and the rabbit was then anaesthetised and bled from the

carotid by means of a canula. The results appeared to show that during the passage through the capillaries some of the inorganic phosphate had been converted into phosphoric ester of the type not hydrolysed by the bone enzyme. Later experiments by Martland and Robison [1924] indicate however that this conclusion is probably incorrect and that the difference between the two samples of blood was due to the shock of anaesthesia and the operation, which caused an increase of the inorganic phosphate apparently at the expense of the ester.

In several experiments the hydrolysis was carried out in duplicate, twice as much bone extract being used in one flask as in the other. The total hydrolysis was nevertheless nearly the same in each although the rates were different. In another experiment after hydrolysis had ceased, a further amount of bone extract was added but no increase of inorganic phosphate was found after a further 24 hours at 37°. The cessation of hydrolysis is therefore not due to inactivation of the enzyme but to the exhaustion of the substrate.

Hydrolysis of the esters by dilute acids.

Zucker and Gutman [1923, 1] have stated that only a part (about 40–50 %) of the organic acid-soluble phosphorus compounds in blood is hydrolysed by boiling with dilute acids and have carried out [1923, 2] a large number of estimations of the amounts of hydrolysable and non-hydrolysable phosphorus in the blood of normal and rachitic rats. The experiments were conducted by boiling the protein-free filtrates for four hours with dilute acids and estimating the increase in the inorganic phosphate. No evidence was adduced that hydrolysis had ceased so that their conclusions seemed to be unwarranted. We carried out some experiments to test the truth of their statement and found that hydrolysis continued, naturally with decreasing velocity, until nearly the whole of the phosphorus was present as inorganic phosphate. The results of one experiment are given below, and are also shown in curve C in the figure. They give some indication of the presence of more than one compound which are hydrolysed at different rates but it is obvious that no estimation of the respective amounts of these compounds could be made from any single point on the curve.

Experiment. The protein-free filtrate from the whole blood of a rabbit was boiled under a reflux condenser with $N/5$ sulphuric acid. The inorganic phosphate in 10 cc. was estimated at intervals.

Time of boiling in hours	Percentage of organic phosphorus hydrolysed	Time of boiling in hours	Percentage of organic phosphorus hydrolysed
½	12·8	12	67·2
1	18·9	16	76·4
2	25·4	20	83·0
4	39·7	36	97·6
8	56·2		

ACTION OF BONE ENZYME ON PLASMA AND ON UNLAKED BLOOD.

Several attempts were made to demonstrate the hydrolysis by the bone enzyme of the acid-soluble phosphoric esters in plasma. These all yielded inconclusive results owing to the very small amounts of these esters present. We then tried to find out whether the inorganic phosphate of the plasma could be increased by acting on the unlaked whole blood with bone extract. The experiment was for several reasons a difficult one to carry out satisfactorily. When blood is mixed with saline alone and kept at 37° for some hours the inorganic phosphate in the plasma is increased. Further it was necessary to keep the p_H at 7·4 and to avoid the use of chloroform or other antiseptic since if any haemolysis occurred the results would be valueless. The duration of the experiment was thus limited by the danger of bacterial growth. Nevertheless positive results were obtained (Table II).

Exps. 1, 2 and 3. Mixed venous and arterial blood of adult rabbits. Potassium oxalate used as anticoagulant.

3 cc. of blood were measured into a number of small centrifuge tubes containing 3 cc. normal saline or 3 cc. of bone extract (made isotonic with blood) or 3 cc. of the same extract previously heated to 100° (in the tube itself). Tubes containing blood alone were also taken. Certain of these tubes were centrifuged at once and the inorganic phosphate estimated in 1 cc. of the undiluted plasma and in 2 cc. of the diluted plasma in the tubes containing added solutions. The remaining tubes were stoppered and kept at 37° for five hours after which they were centrifuged and the phosphate estimated as before.

No haemolysis occurred in any of these experiments.

In Exp. 3 some of the plasma was separated from the corpuscles and was also treated with bone extract, while a control tube containing 3 cc. $M/10$ sodium glycerophosphate solution and 3 cc. bone extract was made up.

Table II.

	Inorganic phosphate (mg. P) in 1 cc. plasma or 2 cc. diluted plasma				
	Time at 37° (hrs.)	Whole blood (alone)	Blood + saline	Blood + heated bone extract	Blood + active bone extract
Exp. 1	0	·050	·032	—	—
	5	·0595	·048	·128	·143
Exp. 2	0	·067	·054	(·088)	·088
	5	·084	·076	·098	·136
Increase		·017	·022	·010	·048
Exp. 3	0	—	·044	·064	·064
	5	—	·056	·080	·092
Increase		—	·012	·016	·028
Exp. 3		Plasma + bone extract	Sodium glycerophosphate + bone extract		
		·062	·020	—	—
		·062	·086	—	—
Increase		—	·066	—	

In every experiment the increase in the inorganic phosphate in the plasma was greatest in the presence of the active bone enzyme which seems to show that the phosphoric esters in the corpuscles were in some way brought into contact with the enzyme and then hydrolysed. The most likely way in which this could take place is by diffusion of the esters from the corpuscles into the plasma, but the experiments do not justify the assertion that this diffusion actually takes place.

Action of the bone enzyme on the lipin phosphorus compounds in blood.

The increases in the amount of inorganic phosphate found in the preceding experiments might possibly have been due to the hydrolysis of phospholipins, although the absence of such increase when plasma alone was treated with the enzyme in Exp. 3 pointed to the opposite conclusion. Robison and Soames [1924] have shown that egg-yolk lecithin is not acted on by the enzyme and the same was also proved for the blood phospholipins by the following experiment.

The blood of two young cockerels was rapidly centrifuged and separated into 43·2 cc. plasma and 38 cc. corpuscles. These were diluted with water and proteins were precipitated with trichloroacetic acid. The precipitates were filtered in Buchner funnels and washed with 100 cc. $2\frac{1}{2}$ % trichloroacetic acid. They were then extracted three times with a mixture of alcohol and ether. After removal of solvent by distillation and re-extraction with ether, the phospholipins were finally shaken up into an emulsion with 50 cc. of water and tested with the bone enzyme with the usual controls. No increase of inorganic phosphate occurred in any of the flasks during 48 hours at 37°, showing that the phospholipins either from corpuscles or plasma were not hydrolysed by the enzyme.

Summary.

The acid-soluble organic phosphorus compounds present in the blood of various animals consist of at least two phosphoric esters, one of which is rapidly hydrolysed by the enzyme of ossifying cartilage, while the other is very resistant to the action of this enzyme.

Both esters are hydrolysed by boiling with 0·2 *N* sulphuric acid.

A method has been worked out by which the respective amounts of these esters (or types of ester) may be determined, and the results of such estimations are given for the blood of human adults and children, and of rats, rabbits and cockerels.

The amount of ester hydrolysed by the bone enzyme varies between 14 % and 36 % of the total acid-soluble phosphorus.

This ester is present almost entirely in the corpuscles, attempts to demonstrate the presence of hydrolysable esters in the plasma leading to inconclusive results.

By the action of bone extracts on unlaked whole blood, the amount of inorganic phosphate in the plasma is increased. This probably indicates that the esters are able to diffuse from the corpuscles to the plasma, and that the former may act as reservoirs.

The phospholipins present in the blood are not hydrolysed by the bone enzyme.

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XCVII. NOTE ON THE ESTIMATION OF PHOSPHORUS IN BLOOD.

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During the course of work carried out in this laboratory during the past eighteen months and involving a very large number of micro-estimations of inorganic and total phosphorus in blood, certain points of interest and sources of error have been noted and are here briefly set out.

Estimation of inorganic and total phosphorus in blood by the Bell-Doisy and Briggs methods.

The original Bell-Doisy [1920] method has been largely superseded by the modification introduced by Briggs [1922] which was free from the main objections raised against the former method. These were the rapid fading of the colour and the diminished intensity of the colour produced in presence of oxalate or citrate. There is another objection, which, so far as we are aware, has not been mentioned previously. The intensity of the final colour varies considerably with changes, in the degree of acidity of the solution in the first stage of the process. This is not so important in the estimation of inorganic phosphate where the acidity of standard and unknown may be equalised, but appreciable errors may occur in the estimation of total phosphorus after acid ignition since the amount of residual acid can only be gauged approximately and may differ from the amount added to the standard. The following examples illustrate the point.

(*a*)	1·0 cc. standard phosphate soln. (0·05 mg. P)			Colorimeter reading	20·0	mm.
(*b*)	,,	,,	+1 cc. 20 % trichloroacetic acid.	,,	20·95	,,
(*c*)	,,	,,	+0·1 cc. conc. H_2SO_4	,,	23·4	,,
(*d*)	,,	,,	+0·3 cc. ,,	,,	28·6	,,

The colour developed in the Briggs modification is, as claimed by its author, independent of the degree of acidity within wide limits. We have found, however, that in estimating total phosphorus, after ignition with nitric and sulphuric acids, the addition of the Briggs molybdate solution (2 cc. of which contains 0·3 cc. conc. H_2SO_4), may bring the total acidity dangerously near to or above the upper limit of safety. We therefore prefer to add 2 cc. of a 5 % solution of ammonium molybdate alone, but take 2 cc. of the Briggs molybdate solution as usual for the standards and make up the total volume to 15 cc. in each case. For the acid ignition we use 1 cc. of a mixture of one part by volume of conc. H_2SO_4 to two parts conc. HNO_3 and carry out the ignition in 50 cc. round-bottomed flasks of resistance glass. Silica pebbles are not necessary as there is no danger of loss by spirting.

Using standard phosphate solutions, very low results were at first frequently obtained, and losses by volatilisation or by formation of pyrophosphoric acid as suggested by Baumann [1922], were suspected to be the cause. Experiments failed to confirm these suspicions, and the losses were finally traced to over ignition, resulting in some of the phosphoric acid being taken up by the glass. The errors were entirely avoided by using more moderate temperatures and stopping the ignition as soon as the white fumes of SO_3 appeared.

Error caused by trichloroacetic acid.

That trichloroacetic acid may be a source of error in the Briggs method was first suspected in the course of experiments reported by Kay and Robison [1924], where double the usual amount of this acid happened to be present in the solutions in which the inorganic phosphate was being estimated.

Investigation showed that trichloroacetic acid produces a blue colour with the Briggs reagents in complete absence of inorganic phosphate. The colour is, however, produced very slowly, and with the customary amounts of trichloroacetic acid used for the precipitation of blood proteins the error thus caused is very small, provided that the colorimetric readings are made not more than 30 minutes after adding the reagents. The following results show the amount of the error under varying conditions and prove also that it is not due merely to the presence of traces of phosphate in the trichloroacetic acid. If this were so, the error would be the same whether the readings were made after 30 minutes or several hours. The increase is however progressive, being inconsiderable at the end of half an hour and rising to very large amounts at the end of 29 hours. This indicates that the reduction of the molybdic acid is catalytically accelerated by the trichloroacetic acid in the same way as by the phosphoric acid, though in a much lower degree[1].

The intensity of the colour developed by the trichloroacetic acid appears to vary with the amount of phosphate present.

Apparent amounts of inorganic phosphate (mg. P) estimated in presence of varying proportions of trichloroacetic acid.

Amount of phosphate added	0				0·050 mg.			0·100 mg.	
Amount of 25 % trichloroacetic acid solution added	0	·5 cc.	1 cc.	2 cc.	·5 cc.	1 cc.	2 cc.	·5 cc.	1 cc.
Read after ½ hr.	0	·002	·0025	·003	·051	·053	·058	·102	·104
1½ hrs.	0	·0035	·0045	·007	·056	·062	·070	·108	·112
3 hrs.	0	·006	·0095	·014	·075	·086	·103	·127	·144
29 hrs.	0	·039	·061	·104	·105	·143	·222	·161	·200

The possibility of errors due to this cause is the more serious because the permanence of the colour is one of the advantages of this method and may lead to the practice of taking the readings after fairly long intervals.

[1] (*June* 20th, 1924.) The source of this error has been traced to an impurity present in all samples of trichloroacetic acid that have been used. The acid was obtained from the B.D.H. and distilled at an almost constant temperature. The small amount of residue contained the whole of the impurity and gave an intense coloration with the Briggs reagents, while the distillate gave no colour at all in 4 hours. The colour-producing substance was destroyed on ignition and is therefore apparently not inorganic.

The increase of inorganic phosphate in the blood after withdrawal from the body.

The increase in the inorganic phosphate which occurs in blood after withdrawal from the body has been frequently noted. It was important to discover under what conditions this increase takes place and by what means it can be prevented. On these points answers were provided by the following experiments.

Estimations of inorganic phosphate were carried out by the Briggs method on different portions of the same whole blood after treatment in various ways as described below. Except where otherwise stated the estimations were made as soon as the trichloroacetic acid filtrates were obtained (about a quarter of an hour after precipitating the proteins) and the colours were matched 30 minutes after adding the reagents. Trichloroacetic acid is referred to as "t. acid."

Exp. A. Mixed venous and arterial blood of rabbit, containing potassium oxalate.

Exp. B. Blood from ear vein of a rabbit, containing potassium oxalate.

Treatment of blood.

		Inorganic phosphate (mg. P) per 100 cc.
Exp. A.	1. Laked with water (3 vols.) and proteins precipitated with t. acid (final conc. 2·5 %) within a few minutes of withdrawal of blood from animal	6·2
	2. Phosphate estimated in the same acid filtrate as No. 1, but after this had remained 5 hrs. at room temperature ...	6·2
	3. Laked at once; laked blood kept 3 hrs. at room temperature before adding the t. acid	8·8
	4. Unlaked blood kept 3 hrs. at room temperature, then laked, and t. acid immediately added	6·3
	5. Same as No. 4, but unlaked blood kept 6 hrs. at room temp.	6·3
Exp. B.	1. Blood laked by running it directly into a 1 % solution of t. acid (3 vols.). After 5 minutes proteins precipitated by addition of more t. acid to make final concentration 2·5 %	5·3
	2. Laked with water; t. acid added after 5 mins.	5·8
	3. Laked with water; t. acid added after 2 hrs. at room temp.	7·9
	4. Laked with water and kept at 37° for 2 hrs. before adding t. acid	9·7
	5. Laked with 1 % t. acid; the rest of the t. acid added after 2 hrs. at room temp.	5·3
	6. Laked with 1 % t. acid and kept at 37° for 2 hrs. before adding the rest of the t. acid	5·3

These results show that the amount of inorganic phosphate in whole blood rapidly increases after the blood has been laked with distilled water. In laked blood kept at room temperature the increase amounted to 10 % in five minutes and 50 % in two hours. At 37° the increase was considerably greater. No increase at all occurred even in two hours at 37° in presence of 1 % trichloroacetic acid, which indicates that the change is due to enzymic hydrolysis.

In unlaked blood kept at room temperature only a very slight increase occurred in six hours, but experiments reported by Kay and Robison [1924] show that when unlaked blood is kept for five hours at 37° the amount of inorganic phosphate in the plasma is increased by about 20 %.

The practical conclusion to be drawn from these facts is that if in estimating inorganic phosphates in blood even a few minutes are allowed to elapse between laking and precipitation of proteins the results obtained will be too high. Wherever possible we now pipette the whole blood directly into three or four volumes of 1 % trichloroacetic acid.

These changes and the nature of the ester and of the enzyme concerned are being further investigated.

The increase of inorganic phosphate in blood as a result of ether anaesthesia and surgical shock.

As stated elsewhere [Kay and Robison, 1924], considerable differences were observed in the amount of inorganic phosphate in blood taken from the ear vein of a rabbit and that taken half-an-hour later from the carotid after anaesthesia. We suspected that this difference might be partly or wholly due to the effect of shock, and carried out some experiments to test this, using the precautions mentioned above.

	Description of blood	Inorganic phosphate (mg. P) in 100 cc. whole blood	Total phosphorus (mg. P) in 100 cc. whole blood
1 *a*.	Human, venous, from med. basilic vein	2·56	—
b.	Human capillary from finger of same subject taken immediately after 1 *a*	2·60	—
2 *a*.	Rabbit, venous (ear vein)	3·03	—
b.	„ „ after 10 mins. ether anaesthesia	3·37	—
3 *a*.	Rabbit, venous (ear vein)	2·62	25·9
b.	„ „ after 15–20 mins. ether anaesthesia	3·90	28·0
4 *a*.	Rabbit, from ear vein	2·86	25·4
b.	„ „ left ventricle	2·86	26·0
c.	„ „ ear vein ½ hr. after cardiac punct.	3·95	27·2
d.	„ mixed blood from carotid and jugular taken at death shortly after *c*.	5·52	30·0

These results show that the amount of inorganic phosphate in whole blood is increased after ether anaesthesia and also as the result of shock consequent upon cardiac puncture. As to the cause of this increase we are not prepared at present to give an opinion. In experiments 3 and 4 the amount of total phosphorus was also increased, which differs from the result noted by Kay and Robison [1924]. The possibility of differences existing in blood before and after passage through the muscles is not of course excluded, though the single experiment (No. 1 of the above series) suggests that such is not the case.

From what has been stated above it will be seen that there are many pitfalls in the determination of phosphorus compounds in blood, and that though under normal conditions the consequent errors may not be very large they may be very considerably increased if the conditions are but slightly changed.

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